HETEROGENEITY IN HUMAN GLIOMAS.

In vitro and in vivo studies of astrocytic neoplasia.

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"These simple tenets have become the sine qua non of modern science. A man who believes that he can empty the jar of the universe and discover all of its secrets is insufficiently humble to engage in modern science. On the other hand, a man who remains unconvinced of the uniqueness and value of his own experience will lack the ego of a scientist."

Charles A Whitney. The Discovery of our Galaxy. Angus & Robertson 1972 London

PREFACE

This thesis gives an account of original research work carried out between January 1984 and July 1987 in the Neuropathology laboratory of the Department of Pathology in the University of Leeds. Sections of the work presented have been published as substantive or abstracted papers included in the list of references at the end of the work.

Chapter 2. In vitro studies of glial tumours - Franks and Burrow, 1986

Chapter 3. Tissue plasminogen activator activity in human gliomas - Franks and Ellis, 1989

Chapter 4. MHC class II antigen expression in gliomas - Franks and Bird, 1986a

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My family, as always, have had to bear the greater burden during the carrying out, and writing up, of the research described. My thanks go to them for their support and understanding.

DECLARATION:

I, Antony John Franks, declare that, with the exception of the technical assistance acknowledged above, the work described in this thesis is entirely my own and the thesis has been composed by myself.

ABSTRACT

The phenomenon of heterogeneity in neoplasia and its implications for tumour growth and behaviour are considered, firstly for tumours in general and then for tumours of astrocytes in particular. Attention is drawn to the nature of cellular interactions that may occur within tumours between populations of neoplastic cells whose differences may impose an interdependence that will determine and control growth. Such differences will also influence the degree to which isolated cells and the properties they display in vitro will be representative of the populations of cells that comprise the tumour in vivo. An account is given of in vitro studies of a series of human astroglial neoplasms with the description of two main cells types derived during such studies: one which appeared to be glial in origin, was process-forming and expressed Glial Fibrillary Acidic Protein, and the other, of uncertain origin, was flattened and adherent in configuration and expressed surface fibronectin. The factors determining the isolation of these two cell types are considered and the conclusion is reached that technique of culture is a more significant determinant of the predominant cell type isolated than is the degree of malignancy of the original tumour. The evidence for and against a glial or mesenchymal origin for the fibronectin-expressing cells is considered.

Studies are then described of the distribution in vivo of a range of phenotypic markers characterising the cells

isolated in vitro. On the evidence provided by this work the fibronectin-expressing cells seem more likely to have originated from vascular, or vascular-associated structures, but may have been influenced by tumour-derived products in such a way that their behaviour in vitro suggests a transformed nature.

To examine other influences on the vascular and glial elements in astroglial tumours studies of immune cell infiltration, growth factor receptor expression and tumour cell growth were undertaken. These indicated that there are qualitative similarities, but quantitative differences between high and low grade tumours that might explain the relative uniformity of the in vitro behaviour of vascular-derived elements.

The hypothesis is advanced that the process of carcinogenesis in astroglial tumours differs from that in many epithelial tissues by virtue of the exposure of vascular elements to any putative carcinogen. Thus vascular components in astrocytic tumours may already have suffered damage that, whilst insufficient to result in neoplastic change, affects their behaviour in vivo and in vitro. In vitro this results in the isolation of cells that appear to be transformed but are not of glial origin, and in vivo in an degree of vascular proliferation unusual in other sites and an increased incidence of sarcoma development.

In vitro studies of human gliomas would be of potential value for examining the interactions between neoplastic

parenchymal cells, mesenchymal elements and cells of the immune system which may be of importance in other neoplasia.

CONTENTS

4.IMMUNE ELEMENTS IN HUMAN GLIOMAS - HETEROGENEITY OF RESPONSE AND TUMOUR ANTIGEN AND GROWTH FACTOR RECEPTOR EXPRESSION.

CHAPTER 1. HETEROGENEITY IN TUMOURS

INTRODUCTION

Heterogeneity is observed in tumours of most types. This chapter examines the phenomenon as it occurs in tumours in general and, specifically, in the subset of gliomas derived from astrocytes. Tumours have three main components, or elements, namely: the neoplastic cell comprising the tumour parenchyma; the mesenchymal tissues that form the tumour stroma; and the mixture of lymphocytes and macrophages that result from the immune response to the abnormal tissue formed by the tumour. All of these elements show heterogeneity and will be considered in the work presented in this thesis.

The nature and possible basis of tumour heterogeneity.

One consequence of biological variation and the essential nature of neoplastic transformation in mammalian cells is that two tumours which are histologically similar will rarely be biologically identical. The cells that comprise individual tumours have been recognised to be heterogeneous in terms of morphology (Foulds, 1954; Shapiro et al, 1981), chromosome content and cell kinetics (Shapiro, 1986; Dexter et al, 1982; Friedlander et al, 1984), antigenic expression (Greiner, 1986; Heppner et al, 1983; Sainsbury et al, 1987), capacity to form tumours from single cells (Franks, 1983; Simnett, 1981), metastatic potential (Aulenbacher et al, 1984; Franks et

al, 1975; Layton et al, 1984), chemosensitivity (Shapiro et al, 1985; Yung et al, 1982) and the influence of population cell density on cell growth (Westermark, 1973).

The concept of a tumour being a mass of tissue composed of a homogeneous population of cells, each individual being autonomous and independent is therefore too simplistic to be of value; yet this is a tacit assumption which often underlies techniques where cells isolated in-vitro are used to test the potential of therapeutic agents (Kimmel et al, 1987; Morgan et al, 1983; Rosenblum et al, 1982).

It is apparent that tumours will consist of a mixture of cells demonstrating a range of properties. These may reflect either varying degrees of damage sustained as a result of exposure to an exogenous carcinogen or further changes in genotype as a consequence of the genetic instability that is known to be a feature of the transformed state (Kendal et al, 1987; Nowell, 1986). The picture is further complicated by the inclusion of normal tissue elements (or their derivatives) in the form either of a supporting stroma or residual normal cells surrounded by invading neoplastic cells.

A polyclonal origin has been demonstrated for some experimental tumours (Reddy et al, 1980) and the multifocal nature of some human skin and urothelial tumours suggests such a derivation. Nevertheless the

current consensus remains that tumours arise by neoplastic transformation of a single cell (Nowell, 1986) with the subsequent emergence of a heterogeneous population, although this ignores the possibility that other cells may be damaged by carcinogenic agents without developing recognisably neoplastic properties.

The mechanisms whereby heterogeneous populations of cells develop in tumours are ill-understood. There is evidence of intrinsic genetic instability manifest as an enhanced mutation rate (Cifone et al, 1981; Kendal et al, 1987; Loeb et al, 1974), possibly mediated by proto-oncogene amplification (Lancet, 1987); it has also been proposed that fusion of tumour cells with reactive cells of the monocyte / macrophage series may occur (Munzarova et al, 1987) thereby conferring properties of invasion and metastasis. Cells whose new properties, however acquired, are not disadvantageous, would be expected to survive, while those with properties which constituted a positive advantage might be expected to form an increasing population within a tumour. A reduced dependence on exogenous factors, or a reduced sensitivity to controlling influences would both confer a major advantage; consequently, with time, cells might be expected to emerge which demonstrated a higher rate of proliferation or greater capacity for survival in areas separate from the tumour mass (i.e. to metastasise). The sequential acquisition of properties, such as invasion or metastasis, is observed in many tumours and is referred to as

progression. It is believed to underly the well-recognised transitions from benign colonic adenoma to invasive adenocarcinoma (Morson, 1974), and from low grade astrocytoma to malignant glioblastoma (Cairncross, 1988). The fact that the chromosomal abnormalities found in low grade astrocytomas resemble in pattern, if not frequency, those of malignant astrocytomas (Rey et al, 1987, 1987a) is strong evidence that such progression does occur.

Another basis for heterogeneity may be the retention and exhibition by tumour stem cells of a potential for differentiation. Thus, hypothetically, some of the structural variation observed in tumours may reflect the various stages of maturation which, in turn, indicate a persistent responsiveness of neoplastic cells to those factors that control the differentiation of the normal cell (Sachs, 1984). An example of this phenomenon is the development of quite complex differentiated structures such as keratin "pearls" in human squamous carcinomas. Factors have been described which induce differentiation in neoplastic myeloid cells and reduce their sensitivity to growth factors (Fibach et al, 1972). Glial Maturation Factor (so named for its effect on normal embryonic glial cells) has been shown to enhance contact inhibition, and specialised intermediate filament expression, in neoplastic glial cells in vivo and in vitro (Lim et al,1986). The maturation of a neuroblastoma into a ganglioneuroblastoma and ultimately into a benign

ganglioneuroma (Willis, 1958) reflects the natural pattern of maturation of neuroblasts. An extreme example of the capacity of stem cells to produce diversity through differentiation is seen in teratomas where a single neoplastic cell is capable of producing a wide range of cell types many of which are fully differentiated (Kleinsmith et al, 1964).

The significance of tumour heterogeneity in the growth of tumours

Research over many decades into the nature of the neoplastic process has seen an increasing realisation that the behaviour of the cells in both natural and experimental neoplasms is more a reflection of the inappropriate expression of certain properties of normal cells than the expression of properties unique to the cancer cell. The behaviour of a tumour may therefore represent the combined behaviour of heterogeneous cell populations rather than reflect the behaviour of any one cell "species".

Variations in degree and combination of these expressions underly the spectrum of behaviours that are designated as benign (small deviations from normal) or malignant (large deviations from normal). It follows from this that while tumour cells undoubtedly express some properties inappropriately, the fact that they also continue to express a great number of appropriate properties offers the hope that they may remain responsive to at least some

of the controls that influence normal cell behaviour. This possibility is seen to be all the more likely when it is appreciated that the transformation of a proportion of cells in a tissue into neoplastic cells is likely to be accompanied by damage to other cells, in the same tissue, that falls short of full neoplastic change. The processes of normal tissue maintenance and response to injury (Simnett, 1981; Riley, 1981) are mediated by cellular interactions which control cell proliferation and migration. There is no reason why these interactions should not persist, at least in part, in tissues in which only a proportion of the cells are abnormal (Heppner et al, 1983) even if the balance results in continued and (from the point of view of the organism) inappropriate growth. One possible mechanism of neoplastic transformation could be a reversal of responsiveness to environmental controls so that what had previously acted as an inhibitory stimulus now elicited a proliferative response. One could hypothesise that if interactions occur between neoplastic cells and normal cells within a tumour this possibility could be greater if they shared a common lineage; this situation is commonly encountered in human gliomas where non neoplastic glial cells may be intimately mixed with neoplastic cells.

The inhibition of non-neoplastic cells by tumour cells (Fontana et al, 1985), and inhibitory and stimulatory interactions between populations of neoplastic cells

(Heppner et al, 1983) have been demonstrated experimentally. Such interactions have been shown to determine the expression of surface antigens and to thus affect the immunogenicity of a tumour and the efficacy of any host response (Wang et al, 1982).
The development by tumour cells of the capacity to invade tissue and metastasise is of importance in the progression of the neoplastic state from one in which cells are confined to an epithelium (in-situ carcinoma). The expression by neoplastic cells of plasminogen activator (PA), which is thought to be involved in the processes of invasion and metastasis can be shown to be influenced by intratumoural cell interactions (Kyner et al, 1978; Newcomb et al, 1978).

The metastatic potential of a tumour has been shown to reside in only a small proportion of its cells (Franks, 1983) and the ability to establish metastases requires not only a capacity to penetrate blood or lymphatic channels but also the ability to survive and grow at low population densities. The latter property is measureable in clonogenic assays (Guner et al, 1977) and the relative infrequency with which cells from human tumours are capable of growth in these low densities emphasises that most tumour cells need specific environmental conditions for continued survival and growth.

Modulation of tumour cell phenotype - possible mechanisms.

The phenotype of any cell should be seen as the result of an interplay between its intrinsic potential to express characteristics and the environment in which it is situated. Gene expression will determine the cell's ability to alter the environment, while the environment, and specifically the extracellular matrix which may be seen as an extension of the cell, will, in turn, modulate the pattern of gene expression (Bissel et al, 1987; Rutka et al, 1987). Consideration of examples from non-neoplastic states such as osteoclastic resorption of bone, trophoblast invasion, epithelial regeneration after injury and vascular proliferation in granulation tissue emphasises the fact that there are, under normal circumstances, interactions between environment and cells which control cellular function and are reflected in alterations in cell phenotype. Any examination of tumour cell phenotype must take account of the fact that such alterations can take place.

Tumour antigens differ quantitatively rather than qualitatively from those of normal cells, and not only is expression heterogeneous within a given tumour but it is often not consistent for a given tumour type (Smith et al, 1988). Focal expression may be found in tumour sections and the same antigen may show cytoplasmic or surface distribution in different cells (Greiner, 1986). Modulation in tumours may reflect a change in cell cycle

times where the expression of a given antigen is a feature of a certain stage of the cell cycle (Kufe et al, 1983) and this may underly the observation that antigen expression may decline with contact inhibition (Horan Hand et al, 1985). Antigen expression is affected by the spatial relationships of cells and thus expression may be found in vivo but not in vitro (Greiner, 1986), and cells that do not express a particular antigen in monolayer culture may do so if allowed to form cellular aggregates in vitro or tumour masses in vivo (Horan Hand et al, 1985). Cells may vary in their sensitivity to antigen-inducing factors such as interferon (Greiner et al, 1985) which may be secreted into the environment, either by neoplastic cells or other elements within the tumour such as reactive lymphocytes or macrophages.
The interesting question is raised of the normal role of many of these surface antigens and their relation to cell proliferation. Whether such antigens are involved in a cell's interactions with the environment (both cells and matrix) by cell recognition (such as the HLA antigens), by acting as receptors for soluble factors or by interacting with extracellular matrix components, they provide a means whereby environmental conditions can affect cell function and conversely a means whereby a cell can fit its behaviour to that environment. There is considerable evidence that the extracellular matrix influences both the growth and differentiation of cells (Toole, 1981; Hynes, 1980; Rutka et al, 1987) and since interactions with the

matrix are central to the expression of at least one neoplastic property, namely invasion, an understanding of these interactions is clearly of importance.

If cellular phenotypes are subject to control or alteration by interactions between the different cell populations that make up a tumour how may such control be effected and could it be exploited therapeutically? An understanding of the mechanisms involved might, for instance, allow therapeutic induction of a defined antigen which might enhance the effectiveness of an immunological attack relying on antibodies specific to such an antigen. Two major possibilities exist for mechanisms of such phenotypic control, one mediated through soluble factors and the other depending on cell contact. There is evidence for the former in the observations of induction of surface antigen expression by interferon (Greiner et al, 1985; Carrel et al, 1985; Takiguchi et al, 1985; Lampert et al, 1985) although part of such an effect may be mediated by increasing the numbers of cells that expressed the surface antigen as well as by increasing the amount of expression on each cell. These studies have shown, however, that not all cells in a tumour are capable of responding to inducing agents, not, it is postulated, because of a lack of surface receptors for the agent but because of a defect in the pathways modulated by activation of such receptors. Factors derived from neoplastic cells have been able to induce reversible transformed behaviour in normal cells,

apparently by activation of surface receptors for epidermal growth factor (DeLarco et al, 1978; Stoscheck et al 1986; Todaro et al, 1978) which appears to be expressed in abnormal amounts by some neoplastic cells (Libermann et al, 1985, 1985a; Ozanne et al, 1986). It has also been suggested that non-neoplastic stromal and immune elements in tissues may be the source of abnormal growth factor production which is responsible for subsequent transformation of parenchymal cells (Lancet, 1986). Although neoplastic cells may differ from their non neoplastic counterparts in their sensitivity to diffusible factors they may still be subject to a degree of control by such factors whatever their source.

Studies of both normal and neoplastic cells have demonstrated the existence between cells of communication channels (thought to correspond to the morphological gap junction) which are potentially capable of transferring molecules, thus allowing metabolic co-operation (Furshplan et al, 1968) and possibly controlling cell growth (Lowenstein, 1979). In vitro studies have shown that normal cells can control the growth of transformed cells via a mechanism dependent on such functioning channels (Mehta et al, 1986); in some experimental systems, however, transformed cells can only communicate with each other and not with normal cells indicating a degree of cell specificity for such a process (Enomoto et al, 1984). Although considerable attention has been paid to the

failure of neoplastic cells to respond to high population density by inhibition of proliferation (contact inhibition) (Alberts et al, 1983) less interest has been shown in the the possibility that individual cells in a tumour may be dependent on a certain cell density to proliferate. The degree to which cells are capable of growth in low densities (the ultimate example being the capability of clonal proliferation from a single cell) varies from tumour to tumour but is rarely great, especially in human lesions. If the component cells of a tumour not only interact but are also interdependent then the role of contacts between cells and the influence of cell-derived soluble factors are potentially of considerable importance (Mehta et al, 1986). The reduction in the number of specialised cell junctions that accompanies the progression of neoplastic lesions in the cervical epithelium (Schindler at al, 1982; Shingleton et al, 1968, 1974) may not only reflect the potential for invasion but also the potential for independent survival. The latter would allow the individual cell to survive and proliferate in the lower densities which are likely to be found at the advancing edge of a neoplasm, or in the even lower densities that will exist in an early metastatic deposit. Such independence may be acquired by individual cells by their producing for themselves those factors required for growth which are normally supplied by their neighbours (Lotem et al, 1983). Only with the development of such reduced dependence on cell density can properties

which may have been present for some time (such as reduced substrate adhesion, motility and invasiveness) become manifest as invasive or metastatic tumour. Such dissociation of the property of invasion from that of metastasis is seen in human basal cell carcinomas, meningiomas, astrocytomas and some salivary gland tumours. The relative lack of variation in cellular DNA levels which is found in metastatic tumours in the brain when compared that found in primary glial lesions (Hoshino et al, 1978) supports the concept that metastasis is dependent on the development of a subset, or clone, of cells possessing the necessary properties.

In vitro studies provide evidence that the properties of transformed cells may be in part dependent on environmental conditions and that these cells differ from normal cells in their reactivity or response to external stimuli (Vasiliev et al, 1981). While the transformed state probably reflects a genotypic alteration the expression of phenotype, at least in the early stages of tumour induction, may be conditional on environmental factors. The acquisition of the ability to maintain the transformed state independently (by further genomic change) could be seen as being essential for invasion or metastasis.

Heterogeneity in human astrocytic tumours.

The heterogeneity that is seen in many experimental and naturally occurring tumours is also seen in human malignant astrocytomas and encompasses most aspects of cell morphology and behaviour including the expression of surface antigens (Budka et al, 1985, 1985a; DeMurault et al, 1985; Rettig et al, 1986; Wikstrand et al, 1985) and growth factor receptors (Libermann et al, 1984; Nister et al, 1986), intermediate filament content (Dahl et al, 1986; Eng et al, 1978; Herpers et al, 1984, 1986; Roessmann et al, 1983; Schiffer et al, 1986), susceptibility to differentiating (Bullard et al, 1981; McLean et al, 1986; Frame et al, 1984; Freshney et al, 1986) and chemotherapeutic agents (Rosenblum et al, 1982; Yung et al, 1982), terminal cell density in culture (Westermark, 1973), and both DNA (Hoshino et al, 1978) and chromosome content (Bigner et al, 1986; Shapiro et al, 1985; Rey et al, 1987, 1987a).

If the principle is accepted that tumour cell phenotype, and thus behaviour, is susceptible to environmental influences (in the broadest sense encompassing extracellular matrix, adjacent cells and their secretions) one is led to consider the degree to which the heterogeneity observed in human malignant gliomas is a reflection of inherent or intrinsic variability, and to what extent it is a reflection of variations in the local environment (that is a mixture of stimulatory and

inhibitory influences deriving from cells that are normal, stimulated but un-transformed, partially transformed or fully neoplastic). The possibility that tumour progression is the result of continuous alteration in tumour cell populations in response to these environmental influences, rather than an inevitable consequence of the acquisition of the neoplastic phenotype, raises the prospect of manipulating the environment so as to reduce the rapidity, or even the occurrence, of such progression. This is of especial importance in glial tumours where a low grade lesion may follow a long clinical course with the eventual, but almost inevitable, development of malignant properties (Rubinstein, 1972).

The heterogeneity referred to above has been posited as the explanation for the frequent isolation from human gliomas of cells which express phenotypes not usually associated with glial cells (Kennedy et al, 1987) and which are interpreted as less differentiated. Alterations in dominant populations in vitro as a result of experimental manipulations are interpreted as phenotypic change in individual cells; there is however the possibility that selective growth and survival of subpopulations of cells may underly some of these observations (Freshney, 1985). The use of cells, isolated in vitro from gliomas, to assay the effects of various potential therapeutic agents depends on their being both neoplastic and of glial origin if the results of the

assays are to have any validity. The use of an isolated cell line may provide a greater guarantee that the cells are truly neoplastic but the isolation of such a line removes the element of cellular interaction that may be essential in determining the growth, progression and behaviour of the tumour in vivo.

Human cerebral tumour classification

As a preliminary to a consideration of the nature of cells derived from human gliomas in-vitro it is necessary to give consideration to tumour components in-vivo.

The majority of taxonomic schemes for tumour classification attempt to combine a statement concerning the present understanding of the cell type from which the tumour has arisen (histogenesis), and an assessment of the likely rate at which the tumour will progress in terms of clinical disease (degree of malignancy). Although a case has been made for the inclusion of factors that have a clear bearing on outcome (such as tumour site and patient age) (Burger, 1985; Gilles, 1985) and other features that may require more specialised techniques such as ploidy (Rorke, 1987), morphological classifications remain dominant (Russell et al, 1977).

When considering tumours of a body system, or organ, it is customary to consider the normal tissue elements found in that system or organ and then to delineate the tumours

that can arise from each of these.

In the central nervous system the two most common tumours arise from glial cells (gliomas) and the arachnoidal cap cell of the leptomeninges (meningiomas). Tumours of the glial cells include those derived from the astrocyte (the most common), the ependyma, the choroid plexus and probably the oligodendrocyte, although there is debate as to the true histogenesis of tumours designated as oligodendrogliomas. Less common tumours include those derived from vascular structures (haemangioblastomas), lymphoreticular cells (lymphomas) and tumours composed of primitive cells showing a range of differentiations which probably reflect a neuroglial origin (the so-called primitive neuroectodermal tumours).

Although a number of different tumour types have been studied at various stages of the work presented in this thesis it is predominantly concerned with tumours of astrocytic origin. These tumours encompass a range of clinical types and behaviour but can be most easily understood if seen as spanning a clinical spectrum from benign slowly growing (low grade) lesions to malignant rapidly progressive (high grade) tumours. Many attempts have been made to produce a clinically relevant, biologically credible, easily applicable and reproducible classification of these tumours (Bailey et al, 1926, Kernohan et al, 1949; Ringherz, 1950, Zulch, 1979) but

while there may be broad agreement over the principles this can hardly be said for the details. As a consequence there is confusion over terminology and a brief account of this is necessary to provide a key for the terminology used in this work.

The two extremes of the histological spectrum are readily recognised. The low grade astrocytoma is composed of cells which are easily recognised as astrocytic and show little cellular variation or mitotic activity. Although there may be variation from area to area in the proportions of fibrillary cell processes to cell body, subclassifications depending heavily on such histological criteria are of limited clinical value if tumour site and patient age are not taken into account.

The malignant end of the spectrum is recognised by the combination of cellular anisocytosis often with giant cell formation; nuclear pleomorphism and hyperchromatism; focal necrosis; mitotic activity; and often prominent vascular endothelial proliferation. The term glioblastoma multiforme is often applied to such a tumour. Some classifications attempt to separate such a lesion from other malignant astrocytomas in the belief that there is a difference in cellular origin but since such a distinction is hard to make, both in theory and in practice, it is not made here. A four grade classification can be constructed, low grade tumours being designated Grade I and high grade Grade IV, with two intermediate grades (II and III)

inserted between these extremes. Grade II tumours are recognisably astrocytic and while they may show some cellular variation, mitoses are scarce and neither necrosis nor vascular proliferation are features. Grade III tumours are malignant by the histological criteria of mitotic activity and cellular pleomorphism, and although vascular proliferation may be present necrosis is not a feature.

Such an empirical classification derives its validity from the observation that its categories correlate broadly with clinical outcome (Burger, 1986 ; Fulling et al, 1985; Nelson et al, 1985; Schiffer et al, 1988) but, as with most classifications, its prognostic value for the individual patient may be limited. Studies employing a "points" system which takes semi-quantitative account of vascular proliferation, pleomorphism, mitoses and necrosis have confirmed the collective value of these features in providing an indicator of likely tumour behaviour (Cohadon et al, 1985).

In any consideration of classification the factor of sampling error cannot be neglected. The nature of the lesion and the investigative approaches applied often result in small focal samples on which diagnosis will be based. The variability of these tumours from area to area, referred to above, may result in an inaccurate assignment to grade I instead of II, or grade III instead of IV; the

more important distinction between high and low grade is likely to be less of a problem unless the biopsy derives from the edge of the tumour, where the relationship of the invasive tumour cells to residual normal structures obscures the primary pattern of tumour structure on which morphological diagnosis is usually based.

It will be apparent from the above account that in addition to the differences in the appearance (and by implication behaviour) of high and low grade tumours there are also differences in the appearances of the non-parenchymal (vascular/mesenchymal) elements. The capacity to induce new vessel formation is a feature of malignant tumours, and, indeed, is a prerequisite for progressive growth. The fundamental nature of this process has been emphasised by studies of the angiogenic potential of experimentally induced tumours and of cells transformed in vitro. Such studies have shown that the capacity of altered cells to induce the formation of new vessels may precede the acquisition of the capacity to form tumours (Ziche et al, 1982). The florid formation of new blood vessels, often grossly abnormal in structure, is such a noteworthy feature in human malignant gliomas of the cerebral hemispheres that it forms one of their cardinal diagnostic features; it also raises the question as to whether this reflects an intrinsic susceptibility of the cerebral vasculature to angiogenic substances or the presence in malignant astrocytic tumours of angiogenic

substances that are more potent than those found in other neoplasms. The impact which the mesenchymal component has on the nature and behaviour of the cells that are isolated in-vitro from gliomas has been specifically addressed in the work to be described.

This thesis is based on a series of studies undertaken to investigate heterogeneity in human astroglial tumours by examining the behaviour of human glioma samples in vitro and the possible influences of environment, cell density and population interactions. The composition of non-neoplastic components, specifically proliferating blood vessels and cells of the immune system, was also studied in a attempt to identify the origins of the cells that can be identified in human gliomas in-vitro, and to consider possible interactions between such components and the neoplastic glial cells that comprise the tumour parenchyma.

CHAPTER 2. THE NATURE AND ORIGIN OF THE HETEROGENEOUS POPULATIONS IN HUMAN GLIOMAS STUDIED IN VITRO

INTRODUCTION

In vitro studies of gliomas

The hope that cells derived in-vitro from human gliomas could be used to investigate the behaviour of the tumour under controlled circumstances and thus predict clinical behaviour and the response of the tumour to potential therapeutic agents (Freshney, 1980; Oktar et al, 1987) provided an impetus to such studies. Such a hope depends on two basic assumptions, namely that the behaviour of individual cells reflects the behaviour of the complex of heterogeneous cell populations that comprise the tumour in-vivo, and that the cells isolated are both neoplastic and representative of the in-vivo population of transformed cells (Pertuiset et al, 1985).

The presence of variant cellular morphologies in explant cultures of human gliomas has been noted (Freshney, 1980; Lumsden, 1971) but only occasionally studied in detail (Gazso et al, 1978). Similar degrees of morphological heterogeneity have been noted in cell lines derived from human gliomas (Bigner et al, 1981; Westermark, 1973) although a dominant pattern tends to occur in each line. That these morphological types are not immutable is apparent from the response of cells to agents which induce a change to a morphology resembling a more differentiated

cell (i.e. with more processes) although there is considerable variation in response to these agents and conflicting evidence of an association between initial morphology and responsiveness (Bigner et al, 1981; Bullard et al, 1981; Freshney, 1985).
Intratumoural phenotypic heterogeneity may reflect an underlying genotypic heterogeneity (Shapiro et al, 1985) or merely local effects on the component cells; the resulting cell populations may therefore reflect the presence of differentiating influences, cell responsiveness to such influences, and differential subpopulation survival or selection in the conditions of culture.

The relative ease with which the cells that result from primary dispersion of human gliomas can be handled and studied has resulted in a relative lack of interest in study of primary tumour explants (Franks et al, 1986; McKeever et al, 1987). The very complexity of their growth patterns and the time required for their analysis have overshadowed the fact that they bear a far closer resemblance to the tumour in-vivo than do separated cells. It is also known that cell aggregates may differ radically from monolayer cultures of the same cells in the expression of surface antigens (Horan Hand et al, 1985). Moreover the admixture of cells that exists in vivo is still present, at least initially, and the subsequent behaviour of these cells in-vitro is more likely to

reflect the influences operating in life.

The ready growth and survival of the cells that can be isolated from gliomas (Ponten et al, 1978) has lead to the not unreasonable assumption that the derived cells are neoplastic. This view is supported by the findings of chromosomal abnormalities (Kennedy et al, 1987; Shapiro et al, 1981) and plasminogen activator synthesis (Frame et al, 1984), the latter being assumed to reflect the finding in both experimental systems and human tumours that plasminogen activator activity is associated with the transformed state (Mullins et al, 1983).

Separation of tumour fragments into their component cells, subgroups of which are selectively cultured, eliminates any possible influence of intratumoural heterogeneity on individual cell behaviour. This, and further changes that may occur in vitro, may invalidate the results of a number of potential therapeutic strategies. These include chemosensitivity testing, antigenic expression (as an index of differentiation), and antigenic specificity for antibody targeting (Lashford et al, 1988; Takahashi et al, 1988) or for the induction of a tumour specific immune response by the injection of tumour derived cells (Bullard et al, 1985).

The heavy dependence upon dissociated cells for such studies necessitates a careful examination of the

relationship which isolated cells bear to the cell populations that constitute the tumour in vivo. Arguments in favour of the use of isolated cells instead of explants include the observation that chromosomal patterns change with time in explants (Shapiro et al, 1981); this could, however, be taken as an indication that natural progression occurs in-vitro, and this would support the use of explants.

The identification in vitro of cells that are intrinsically abnormal and, by implication, neoplastic is usually dependent on the demonstration of an abnormality of growth pattern, an abnormality of relationship to the substrate upon which the cells are growing, or an abnormality of requirements for growth. Although these features are assumed to equate with malignancy they are really a measure of the cells' ability to survive and multiply in a selected environment and there is no single property in vitro that correlates absolutely with the capacity to form tumours in vivo. Sequential studies of cells during induced transformation have shown that although certain features relating to reduced adhesion of cells to their substrate (one marker of transformation) correlated with each other they did not correspond with loss of growth control (Curatolo et al, 1983). The assumption that the presence of properties of transformed cells in a different population identifies them in turn as transformed may not therefore be justified. It has been

shown, in fact, that discrepancies exist between biological malignancy of cells (as assessed by the ability to form tumours in vivo, or actual origin from a naturally occurring tumour) and their behaviour in-vitro (Franks, 1983). This occurs with sufficient frequency to indicate that in-vitro criteria of "transformation" do not necessarily equate with the capacity to form tumours, and by extension should not necessarily be taken to indicate origin from a neoplastic population.

Among the measures most frequently used of in-vitro transformation are growth in soft agar, loss of contact inhibition, growth in low serum concentration and survival on serial transfer. These properties rely on differential survival in culture conditions to identify cells capable of growing in defined environments. Any studies that involve passage will, by definition, destroy those aspects of the microenvironment in a primary explant culture which cell density and cell subpopulation mixture contribute. Without comparison of residual and passaged cells the potential influence of passage in determining the outcome, in terms of the type of cell isolated, may not be appreciated.

Fibronectin and Glial cells

In 1973 Ruoslahti and co-workers described the production by fibroblasts in tissue culture (Ruoslahti et al, 1973) of a surface protein which was subsequently found to be lost following in-vitro viral-induced transformation of cells (Gahmberg et al, 1974; Vaheri et al, 1974; Wartiovaara et al, 1974). Further studies revealed this protein to be a major component of connective tissue matrices and, although variously named Large External Transformation Sensitive protein (LETS) (Hynes et al, 1974) or Fibroblast-Surface Antigen (Ruoslahti et al, 1973), recognition of its fibrous structure and cell binding properties resulting in universal adoption of the name Fibronectin. Fibronectin was found to be secreted by cells in a soluble form which could be polymerised to form an extracellular matrix (Furcht et al, 1979; Hedman et al, 1978).

Ruoslahti later emphasised that the loss observed in transformed cells resulted not from decreased production but from a failure to bind the protein to cell surfaces and to deposit it in a surrounding insoluble matrix, although the surface receptors for fibronectin were retained (Ruoslahti, 1984). This defect in matrix formation could be reversed in virally-transformed cells by dexamethasone (Furcht et al, 1979) or butyrate (Hayman et al, 1980). The binding of cells to their substrate that was mediated by fibronectin was found to further depend on the state of S-S bounds (Ali et al, 1978; Grinnel et al,

1980); reduction of these to -SH groups reduced cells' ability to adhere.

A significant role for fibronectin is as a modulator of interactions between cells and their environment. It has been found to act as a growth factor for fibroblasts (Bitterman et al, 1983), to determine migration (Schor et al, 1981) and growth patterns in-vitro through its close transmembrane relationship with intracellular actin microfilaments (Hynes et al, 1978; Marshall et al, 1978), and similar influences are postulated to operate in embryonic cell migration where reduced surface fibronectin acts as a permissive or facilitating factor (Erickson et al, 1980).

Studies of tissue localisation of fibronectin show that it is widely distributed within connective tissue matrices, in particular in relation to basement membranes (D'Ardenne et al, 1983; D'Ardenne et al, 1984), and it is also localised to the pericyte/endothelial interstitium of the microvasculature (Courtoy et al, 1983).

In the central nervous system fibronectin appears histologically to be restricted to mesenchymal structures (vascular or meningeal) in both normal and neoplastic tissue, including glial tumours (Schiffer et al, 1984; Chronwall et al, 1983; Kochi et al, 1983; Paetau et al, 1980). The finding that many cells derived in short and long term cultures from malignant human gliomas expressed the surface fibrous adhesion protein fibronectin, and not

the typical glial intermediate filament Glial Fibrillary Acidic Protein (GFAP) (Kennedy et al, 1987; McKeever et al, 1987; Paetau et al, 1980a; Ponten et al, 1978; Rajaraman et al, 1978; Schachner et al, 1978; Vaheri et al, 1976) together with the earlier observations that fibronectin was usually less expressed in transformed than normal glial cells (Vaheri et al, 1976) thus seemed to present a paradox. It was also observed that cells expressing fibronectin appeared to be distinct from those that expressed GFAP (Paetau et al, 1980a). Explanations proffered for these findings included a number of possibilities: that glial tumours were composed of a mixed population of neoplastic cells and that culture conditions favoured one subtype (a fibronectin-expressing stem cell); that phenotypic alteration was taking place in vitro (de-differentiation) and a property not evident in-vivo was becoming apparent; that mesenchymal elements were growing from mixed cultures; or even that gliomas were composed of neoplastic mesenchymal elements with a glial component represented by stimulated but non-transformed glial cells.

Against this background an in-vitro study was carried out on a series of human gliomas paying particular attention to the differences in cell type that were isolated under different conditions and the effect that culture conditions had on the make-up of the predominant cell population.

MATERIALS AND METHODS

Biopsy specimens from a total of 71 tumours were studied, consisting of 67 astrocytomas (50 grade IV, 11 grade III and six grade I & II), three gangliogliomas and one subependymal giant-cell astrocytoma. Material from 37 of the astrocytomas (26 grade IV, six grade II and five grade I & II), one of the gangliogliomas and the subependymal giant-cell astrocytoma was studied immunohistochemically after culture.

Tissue culture

Preliminary studies had shown that growth was far better on the plastic surface of tissue culture flasks than on plain glass coverslips and, although coating the latter with collagen did offer some improvement, the autofluorescence of collagen was a major impediment to immunohistochemistry and all subsequent cultures were standardised to plastic flasks.

Tumour specimens, obtained at operation, were immersed in sterile RPMI 1640 tissue culture medium with antibiotics for transport to the tissue culture laboratory. After washing in fresh RPMI 1640 the tissue was divided into the following culture systems:-

1. 3-5 explants of approximately 1 cu mm were placed directly onto the plastic surface of Corning 25cm^2 tissue culture flasks with 5 ml of RPMI 1640 containing

morpholinopropane sulphonic acid (MOPS) 2.62g/l (Sigma) with 10% heat inactivated donor calf serum (Gibco, Paisley, Scotland), penicillin 100 IU/ml and streptomycin 100 µg/ml. The nutrient medium was left unchanged for the first 5-7 days and thereafter changed twice weekly.

2. Cell Suspensions. The remaining tissue was finely chopped and either dispersed by repeated syringing until a uniform suspension of cells was obtained or, if too tough to be treated in this manner, incubated for varying periods at 37°C in 10 mls MOPS buffered saline containing 1mM Ca^{++} and 250µl collagenase. In the early phases of the study Sigma brand collagenase was used but later Worthington brand was used since this was found empirically to result in more consistent growth of separated cells.

Experimentation with the period of exposure to collagenase failed to reveal a consistently optimum period but satisfactory results were obtained with periods as short as one and as long as 72 h. In the case of the longer periods viable cells were obtained by pipetting supernatant cells in suspension at 24 h intervals and leaving the collagenase to act on the residual tissue fragments.

In general, treatment was continued for 24 or 48 h and for longer periods only when sufficient tissue was available. After centrifugation of the suspension at 500G for 5 min the pellet was washed with MOPS buffered saline and

incubated in 4ml phosphate buffered saline with 100µl Dithiothreitol and 50µl Papain for 5 min at 37°C. After washing twice in phosphate buffered saline the pellet was resuspended in nutrient medium in 25cm² tissue culture flasks of the same type as used for explants.

The cultures were examined regularly using a phase contrast inverted microscope which allowed study and photomicrography (using Kodak Ektachrome 50 ASA tungsten film) to be carried out without disturbance. Note was taken of the pattern and amount of growth, and the morphological features of the cells and their relationships to each other.

Primary explant cultures were maintained for up to 41 days and primary cultures of dispersed cells for a maximum of 56 days.
In cultures from 15 tumours the effects of passage on the component cells were studied.

Passage was carried out when an established growth pattern was recognised or when it was apparent that the proliferating cells were approaching confluence. There was considerable variation in the time taken to reach this stage which was usually within 21 days of initiation although it might be reached as early as 7 days. At this point the culture was treated with 0.1% trypsin (Gibco), 0.1% EDTA (Sigma) in Ca^{++} and Mg^{++} free phosphate buffered

saline for 15-30 min and new flasks with fresh nutrient medium were seeded. In a few cases direct observations were carried out on the cells and their behaviour during the process.

The passaged cultures were studied as above and the process repeated up to a maximum of 10 times with a maximum total period in-vitro of 100 days.

Immunohistochemistry

One purpose of this work was to examine the possible effects of culture in determining cellular phenotypes and population mixtures. Therefore the standard devices of studying cells from primary cultures by removing them from their primary culture substrate and depositing them on glass cover slips, or actually carrying out the culture on a substrate designed primarily for microscopy were avoided. Entire cultures in their plastic Falcon flasks were fixed for 15 min in either methanol or a mixture of 95% ethanol/5% ether following five washes in sterile Tris Buffered Saline (TBS). The flasks were then stored at 4°C until required. Areas of interest which had been previously identified by phase contrast microscopy and outlined on the flask base by marker pen, were cut from the flask by means of an electrically heated wire. This method allowed multiple samples, as small as $1cm^2$ to be studied from one flask and had the advantage that histochemistry was carried out on the same specimens that

had been observed by phase contrast microscopy.

Wherever possible double staining was utilised to maximise the information obtained from each specimen.
Most specimens were stained using a four layer technique (see below) combining fluorescein and rhodamine conjugates which could be visualised using a fluorescence microscope with mean excitation wavelengths of 490 and 540 nm. These wavelengths were obtained by a simple filter switching device without disturbing the specimen and thus co-localisation patterns could be studied and photographed (on 3M tungsten film, ASA 400 rated at 3200 ASA to allow short exposure times).

Four layer immunohistochemical technique

All stages were carried out at room temperature.

1. Pre-treatment with normal goat serum (NGS) diluted 1:5 in Tris buffered saline (TBS) to block non-specific binding sites.

2. Stain for 1 h with mouse anti-human monoclonal antibody diluted in NGS (diluted 1:20 in TBS). The dilution of the antibody was determined by prior staining of control sections of tissue appropriate to the antibody. 3. Wash in TBS

4. Goat anti-mouse fluorescein conjugate diluted 1:50 in NGS diluted 1:20 in TBS.

5. Wash in TBS

6. Rabbit anti-human polyclonal antibody applied for 30 min. The dilution of the antibody was determined by prior staining of control sections of tissue appropriate to the antibody.

7. Wash in TBS

8. Swine anti-rabbit rhodamine conjugate (diluted 1:50 in TBS) for 30 min.

9. Wash in TBS

10. Mount in citifluor or glycerol/TBS 1:9 with glass coverslip cut to a size appropriate to the specimen.

In a number of preparations steps 2 to 4 were replaced by a 30 min exposure to Ulex europaeus lectin conjugated to FITC (diluted 1:50 in phosphate buffer) followed by steps 5 to 10 as above. All washes and dilutions were with Phosphate buffer at pH 6.8 rather than TBS.

Identification of proliferating cells.

Tumour growth in vivo can be studied indirectly by counting mitotic figures in a section (which will include only that proportion of dividing cells actually in an identifiable stage of mitosis) or by the use of antibodies to nuclear antigens which are only expressed by proliferating cells irrespective of their stage in the cell cycle. Such a protein was identified by a monoclonal antibody Ki67 (Gerdes et al, 1983) which was raised during a search for a marker specific to Reed-Sternberg cells. The protein was found to be expressed on chromosomes from all stages of the cell cycle and in interphase cells, but not in G0 cells. Subsequent work validated these findings (Gerdes et al, 1984) although G1 expression was variable and depended on whether the cell was entering the cycle for the first time (in which case it was not expressed). The antigen was thought to be bound to DNA and possibly a histone necessary for DNA synthesis (Sasaki et al, 1987). The use of this antigen as a marker of cell proliferation in human tumours (as opposed to experimental cells in vitro) gained credibility with the finding that counts of cells expressing it histologically in breast tumours correlated with other conventional markers of grade of malignancy such as nuclear morphology, mitotic activity and degree of differentiation (McGurrin et al, 1987). Consequently commercially available Ki67 was used to identify cells in a proliferating state in vitro.

Binding of Ki-67 was demonstrated by a peroxidase method combined with a second polyclonal antibody (anti-fibronectin) demonstrated by a fluorescent technique. This allowed counts to be made both of Ki67 stained and unstained cells using ordinary tungsten illumination, while still allowing fluorescent identification of a surface antigen that would have obscured the nuclear staining had a standard visible reaction product been used. Since the two antigens being studied were clearly distinguished by their localisation cross reaction of the second conjugate with the first antibody was not considered to constitute a problem.
In these cases the first layer was demonstrated by a rabbit anti-mouse peroxidase conjugate diluted 1:50 for 30 min and subsequently developed with 3'3 diaminobenzidine tetrahydrochloride monohydrate solution with hydrogen peroxidase for 5-10 minutes. The second antibody was demonstrated with goat anti-mouse fluorescein conjugate as step 4 above.

The following antibodies and fluorescent conjugates were used at the dilutions indicated:

Polyclonal

Glial Fibrillary Acidic Protein (GFAP) (Dakopatts; diluted 1:200)

Factor 8 Related Antigen (F8-RAg) (Dakopatts; diluted 1:100)

Fibronectin (FN) (Dakopatts; diluted 1:200)

Monoclonal

Fibronectin (A gift from Dr G Hodges, Tissue Interaction Laboratory, ICRF, London; diluted 1:25) (Trejdosiewicz et al, 1985)

Desmin (Dakopatts; diluted 1:10)

Vimentin (Dakopatts; diluted 1:10)

Ki67 (Dakopatts; diluted 1:5)

Conjugates

FITC goat anti-mouse - Fab2 fragment (ICN; 1:50)

FITC swine anti-rabbit (Dakopatts; 1:50)

Rhodamine goat anti-mouse (Nordic; 1:50)

Rhodamine swine anti-rabbit (Dakopatts; 1:50)

RESULTS

Growth from one ganglioglioma and five grade IV astrocytomas was insufficient for study. Information was therefore gathered from a total of 65 tumours.

There was considerable variation from case to case in the time taken for growth to occur and for the pattern of that growth to become apparent. Histochemical studies were, by definition, associated with the termination of the particular flask studied and so the account that follows is a composite picture built up from many separate specimens, in some cases deriving from the same tumour and observed in parallel, and in some cases from different tumours examined after different periods in-vitro. The majority of specimens were from high grade astroglial tumours and it is on these that the account concentrates. The findings in the subependymal giant cell astrocytoma (SEGCA) and one ganglioglioma differed so radically that they are accorded separate consideration.

Although the cell densities of the suspensions used to set up primary cultures were not quantified it was apparent that low concentrations of cells only rarely gave rise to growing cultures even though the individual cells were capable of survival for many weeks. When collagenase treatment or aspiration dispersal resulted in suspensions containing small intact tissue fragments growth of these

seemed to be enhanced when compared to larger single explants.

Morphology

At an early stage in the study it was apparent that two simple morphological attributes could be used to classify cells growing from tumour explants or cell suspensions. These were the production of long cell processes and the adhesion of the cell body to the growth substrate. These attributes were exemplified in their extreme (and largely exclusive) forms as Process Forming (PF) cells and Flattened Adherent (FA) cells (fig.2.1).

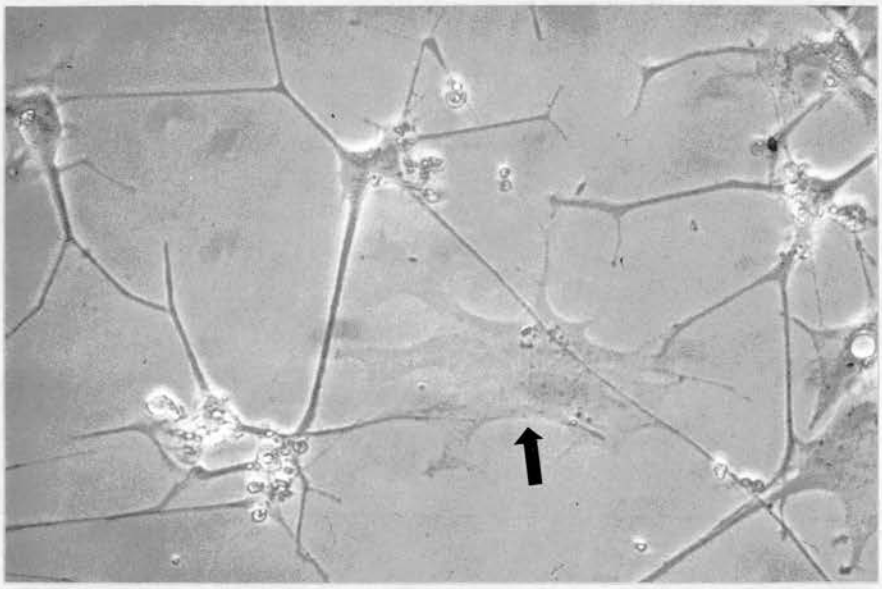
The behaviour of these cell types differed depending on their density and admixture, and although intermediate forms were seen these were usually in defined circumstances (see below) and it was usually relatively easy to allocate a cell to one or other category.

a) Process Forming cells

In low density these tended to be elongate, bi- or unipolar (fig.2.2), and, although, with time, process formation became more complex they did not proliferate, and in older cultures (30+ days) began to degenerate (fig.2.3). These cells consistently stained strongly for GFAP (fig.2.4) and showed vimentin staining that was usually as strong as that for GFAP (fig.2.4) although occasionally only a weak reaction was seen.

Fig.2.1 Typical process forming (PF) cells contrast with single flattened adherent (FA) cell (arrow) near centre of picture. High grade astrocytoma, 21 days in vitro, phase contrast x264

Fig.2.2 Bipolar PF cell from low density dispersed culture, showing adherence points at angulations of its processes (arrows). High grade astrocytoma, 19 days in vitro, phase contrast x264



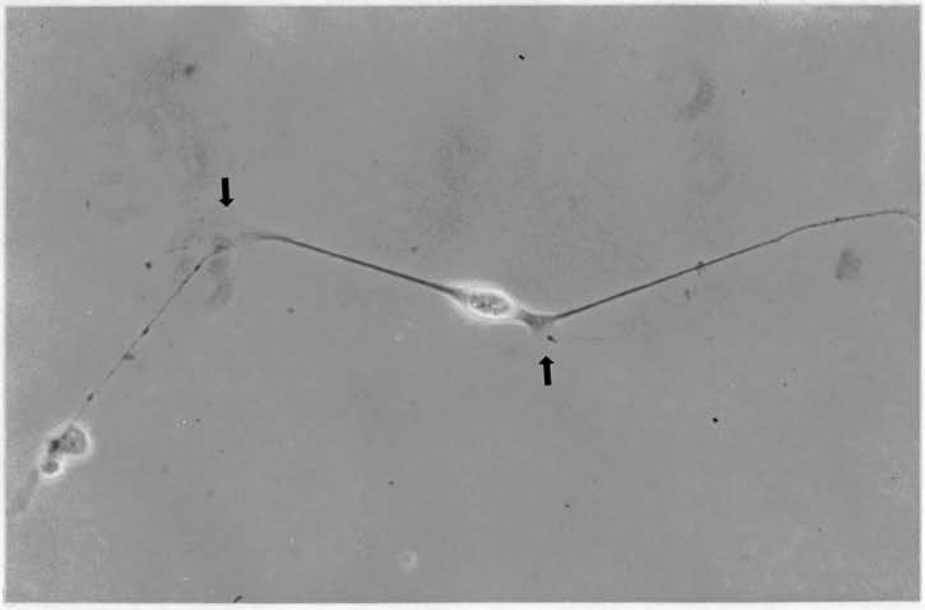
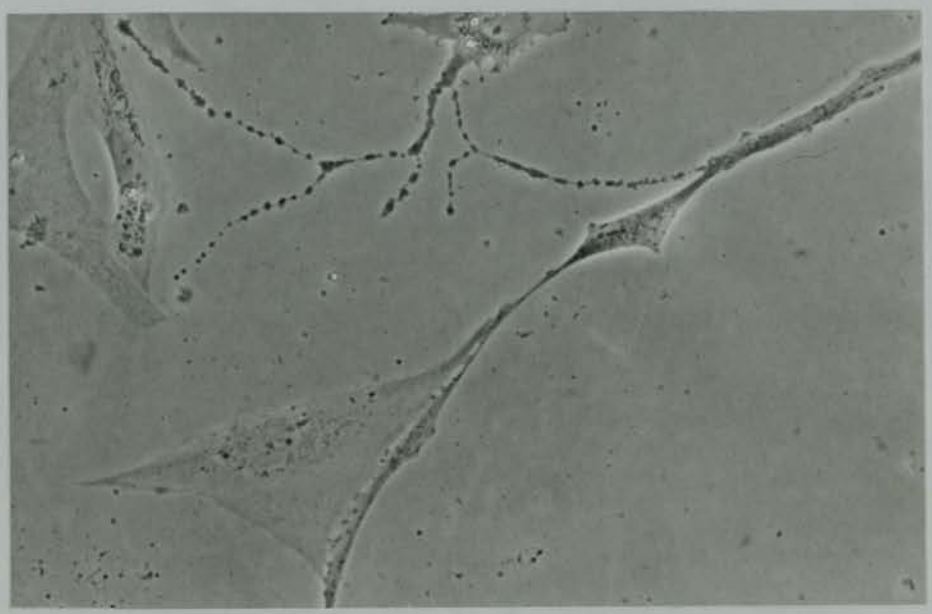
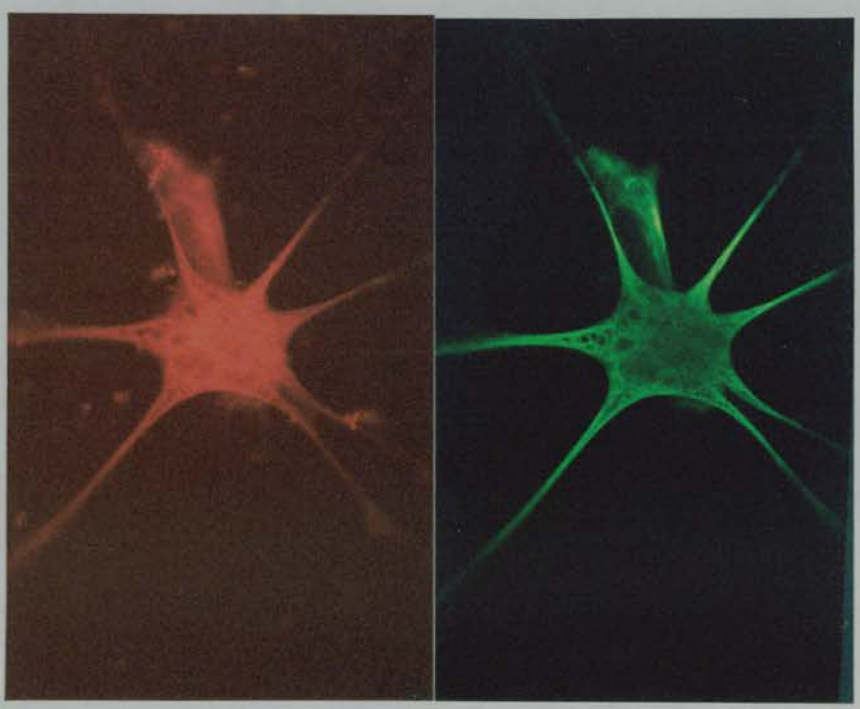


Fig.2.3 Residual FA and PF cells in a low density dispersed culture. The processes of the upper cell are showing granular fragmentation which, together with irregular cell body outline, indicate imminent cell death. High grade astrocytoma, 47 days in vitro, phase contrast x264

Fig.2.4 Multiprocessed PF cell showing uniform staining for GFAP (left) and Vimentin (right). High grade astrocytoma, 41 days in vitro, polyclonal anti-GFAP, monoclonal anti-Vimentin, x260





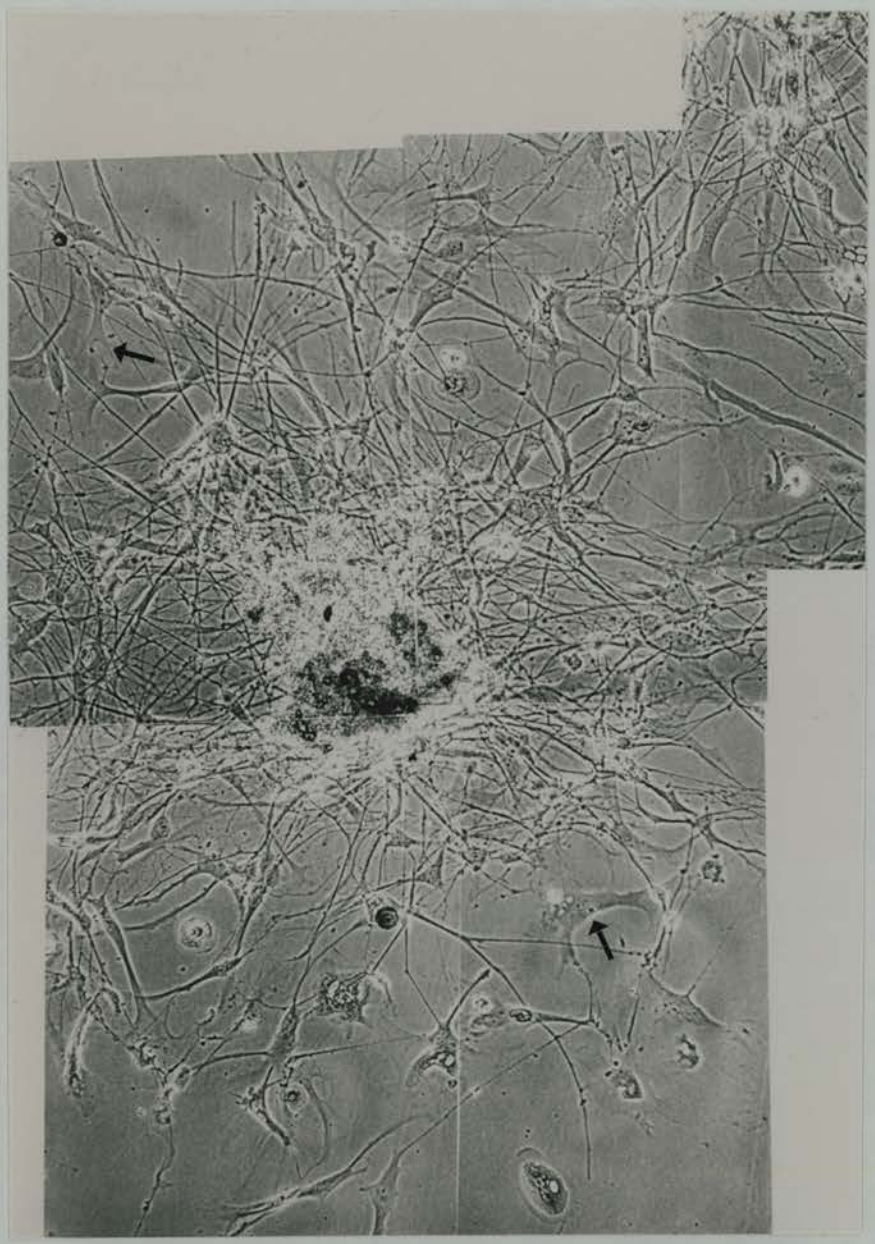
Although such cells in early cultures stained for Desmin (fig.2.5) this was extremely rare in older or passaged preparations.

In high density, typically at the edge of an explant but also in high density cultures from collagenase treated tissue, PF cells formed a dense complex net of refractile processes and cell bodies (figs.2.6 & 2.7), almost invariably associated with FA cells which lay within or beneath the mesh of cell processes in interstitial locations (fig.2.8). PF cells were GFAP+ (fig.2.9) and their processes frequently made contact with FA cells (fig.2.10) and studies of a small number of cultures by time lapse photography showed what appeared to be random extensions and retractions of cell processes before permanent contacts were formed. Only very rarely did PF cell bodies overlie FA cells.

In explants the interstitial FA cells were almost all GFAP+ (fig.2.11) and FN- although occasional single cells were seen that were GFAP-/FN+ (fig.2.12). The process-forming cells invariably stained strongly GFAP+ and usually contained vimentin. FN was occasionally identifiable as small spots of fluorescence on single cells but was never uniform over cell surfaces, and as a result there was an almost mutually exclusive expression of FN and GFAP (figs.2.13 & 2.14).

Fig.2.5 Irregular PF cell showing strong staining for desmin in an early dispersed culture. High grade astrocytoma, 3 days in vitro, monoclonal anti-Desmin, x260

Fig.2.6. Composite view of small explant showing typical complex network of glial cell processes, with smaller numbers of FA cells lying beneath (arrows). High grade astrocytoma, 17 days in vitro, phase contrast, x85



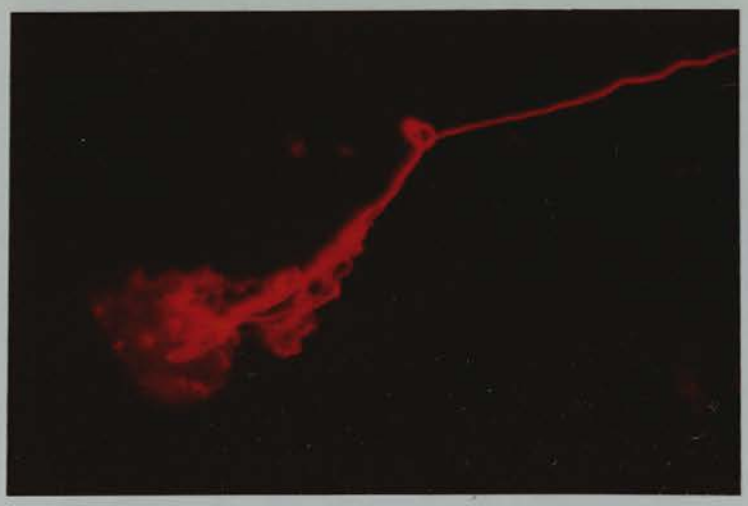
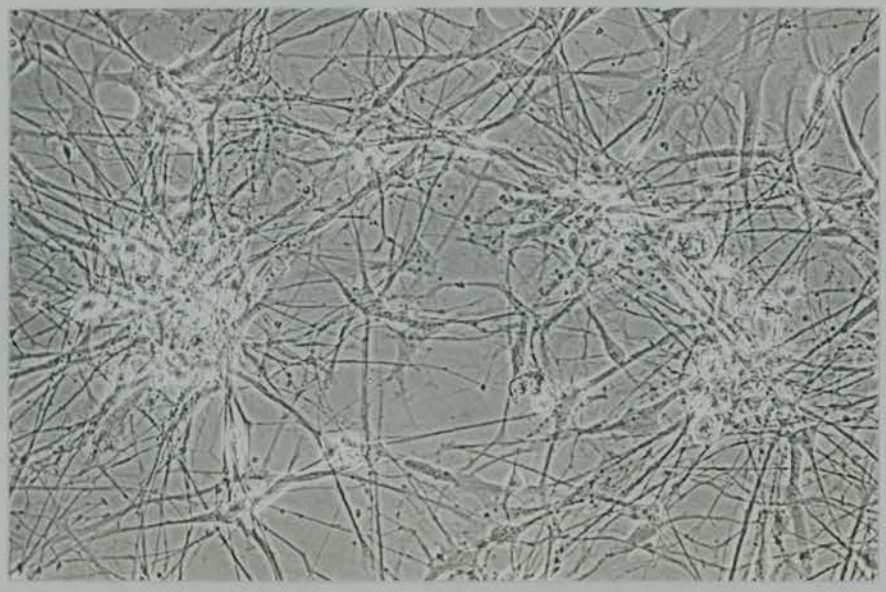


Fig.2.7 Glial network at the edge of an explant culture. Same specimen as 2.6, 17 days in vitro, phase contrast, x132

Fig.2.8 At higher magnification the complexity of glial cell processes is apparent and more detail can be discerned of the relationship between PF cells and interstitial FA cells (arrow). High grade astrocytoma, 17 days in vitro, phase contrast, x264

Fig.2.9 All PF cells and their processes at the edge of an explant contain GFAP. High grade astrocytoma, 20 days in vitro, polyclonal anti-GFAP, x260



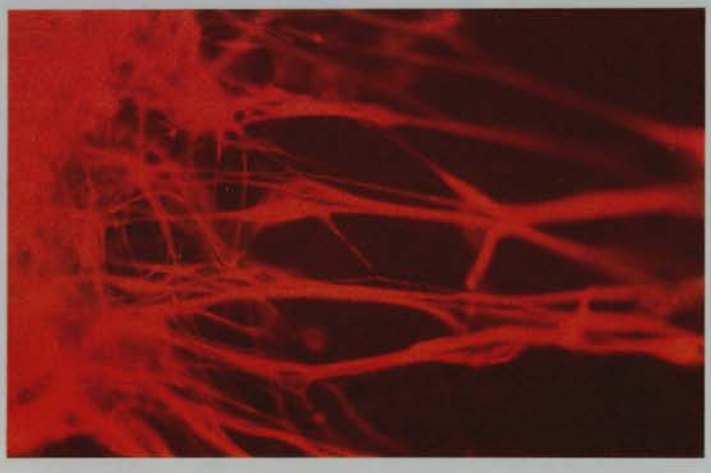
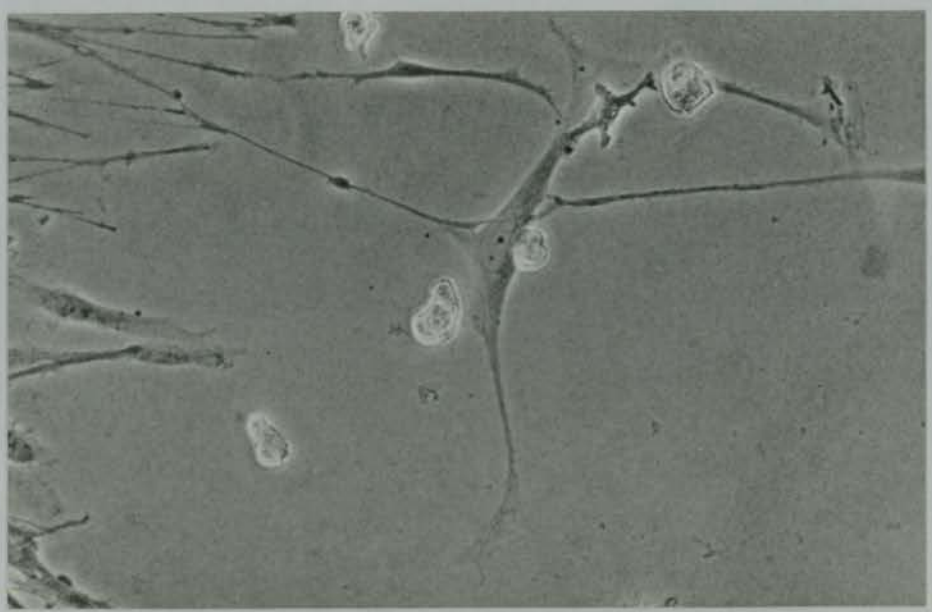
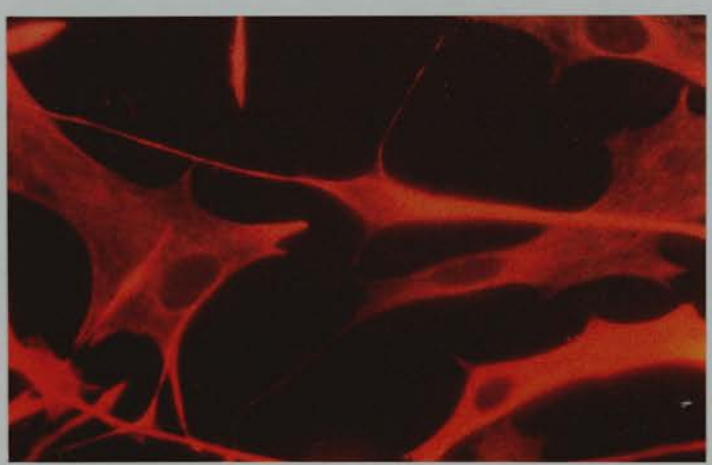


Fig.2.10 Processes extended from PF cells make contact with the cell body of an FA cell (centre). Time-lapse photography showed that some of these contacts were transient. High grade astrocytoma, 6 days in vitro, phase contrast, x264

Fig.2.11 Both PF and FA cells in an explant show uniform staining for GFAP. High grade astrocytoma, 9 days in vitro, polyclonal anti-GFAP, x520

Fig.2.12 Double exposure showing GFAP+ glial network with single FN+/GFAP- cell outlined by its surface staining pattern (arrow). High grade astrocytoma, 21 days in vitro, polyclonal anti-GFAP (orange), monoclonal anti-FN (green), x520





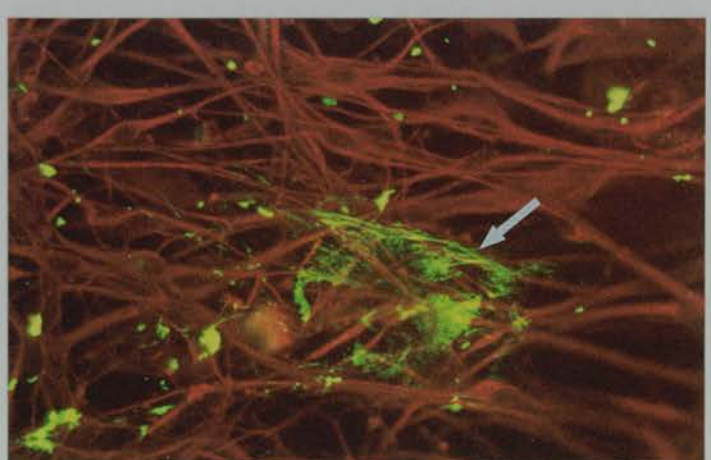
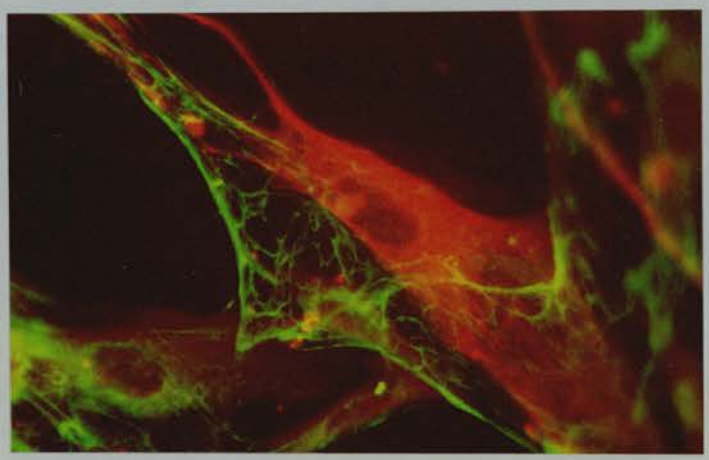
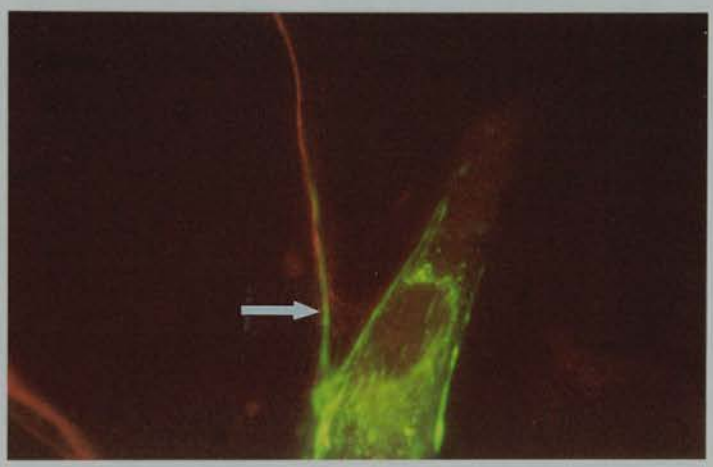


Fig.2.13 Double exposure showing the mutually exclusive expression of FN (green) and GFAP (orange) by cells. High grade astrocytoma, 36 days in vitro, polyclonal anti-GFAP, monoclonal anti-FN, x520

Fig.2.14 In this view of a GFAP+ (orange) PF cell process attached to the body of an FN+ (green) FA cell the extension of fibronectin onto the glial cell process can be seen (arrow). High grade astrocytoma, 31 days in vitro, polyclonal anti-GFAP, monoclonal anti-FN,x520





Where the relative proportion of PF cells remained high a mixed growth continued without loss or degeneration of PF cells. As cell densities increased the space between cells diminished although overgrowing of cells to form heaps above the level of general cell growth was not seen.

In three preparations, consisting largely of PF cells, studied at 15, 16 and 20 days 5.2%, 35% and 9% of PF cells (all FN-) showed nuclear staining with Ki67 (fig.2.15).

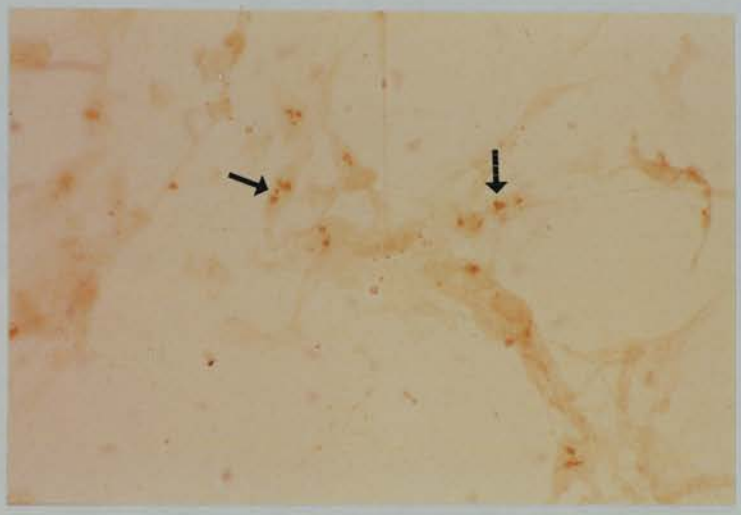
In dispersed cell preparations GFAP+ PF cells dominated in the early days and occasional PF cells were seen that were strongly Desmin+. By 13 days, in the absence of small explant-like aggregations of PF cells, the numbers of single GFAP+ cells had often begun to decline and although at 20 and 31 days they persisted in small numbers they only occasionally had the typical morphology of PF cells and were more usually of FA type (see below) although still FN-.

b) Flattened Adherent cells

In low density large cells of this morphology showed no proliferation and might remain unchanged for the entire culture period. With prolonged culture some cells would develop cytoplasmic elongations, with small numbers of fine refractile processes (fig.2.16). Only very occasionally was a slow transition to a typical PF cell seen.

Fig.2.15 Proliferating PF cells show positive nuclear staining with Ki67 antibody (examples marked by arrows). High grade astrocytoma, 16 days in vitro, Ki67 peroxidase, x167

Fig.2.16 FA cell showing the development of peripheral processes after prolonged culture at low density. High grade astrocytoma, 44 days in vitro, phase contrast, x132



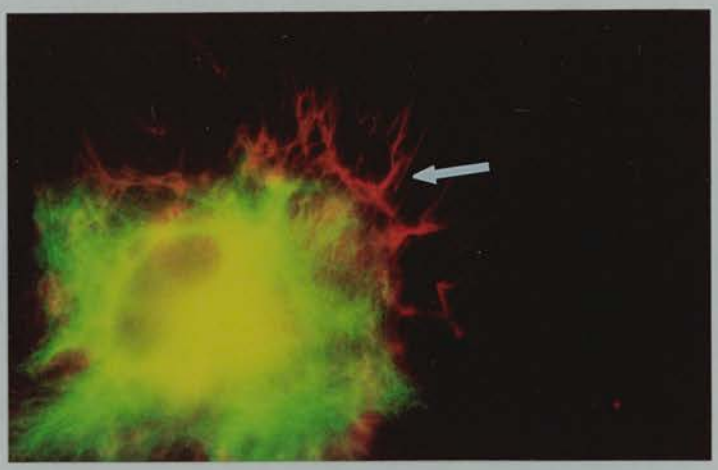
Single large FA cells showed a range of histochemical reactions. The majority stained strongly for surface FN and intracellular vimentin (fig.2.17) with no GFAP or Desmin. Some cells however showed a pattern of strong GFAP positivity with variable vimentin staining and no FN (fig.2.18). The latter were interpreted as isolated cells of the type forming the interstitial cells seen in explants (described above).

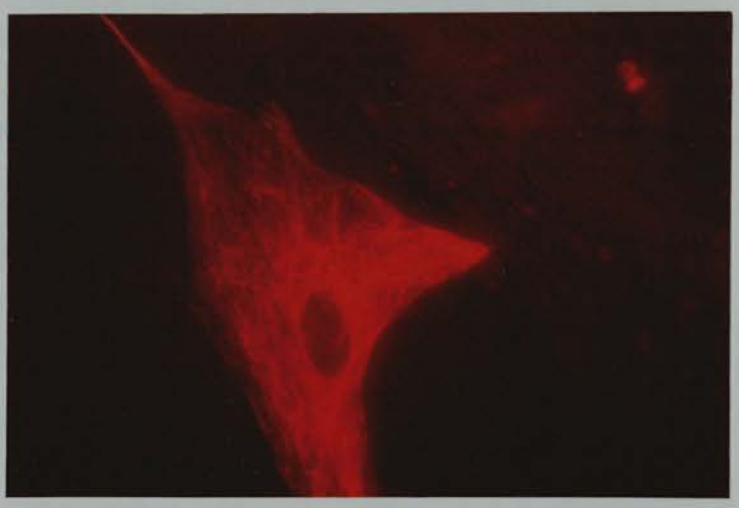
Where FA cell numbers increased, a phenomenon often first observed at the advancing edge of an outgrowth, they began to heap up before reaching confluence. In many cases the appearances suggested the "escape" of cells from the edge of an established PF cell explant (figs.2.19 & 2.20), although sometimes FA cells formed the sole outgrowth from small explants. PF cell progression over these denser FA areas diminished as did the contacts between PF cell processes and FA cells that were a feature of intra-explant growth. This would be followed by progressive loss of overlying PF cells which showed process retraction, rounding up and cytoplasmic granulation (figs.2.21 & 2.22). That this was not simply the result of prolonged culture was apparent from the observation that of two adjacent explants one might show persistent mixed growth and maintenance of a complex network, while the other would show PF cell loss associated with increasing numbers of FA cells (fig.2.23).

Fig.2.17 FA cell with strong staining for vimentin (green) shows extension of its surface fibronectin (orange) onto the adjacent substrate (arrow). Polyclonal anti-FN, monoclonal anti-vimentin, High grade astrocytoma, 56 days in vitro, x520

Fig.2.18 FA cell showing intracellular GFAP+ cytoskeletal filaments. High grade astrocytoma, 36 days in vitro, polyclonal anti-GFAP, x520

Fig.2.19 FA cells (arrow) grow from the edge of a typical glial explant (top left). Recurrent High grade astrocytoma, 7 days in vitro, phase contrast, x264





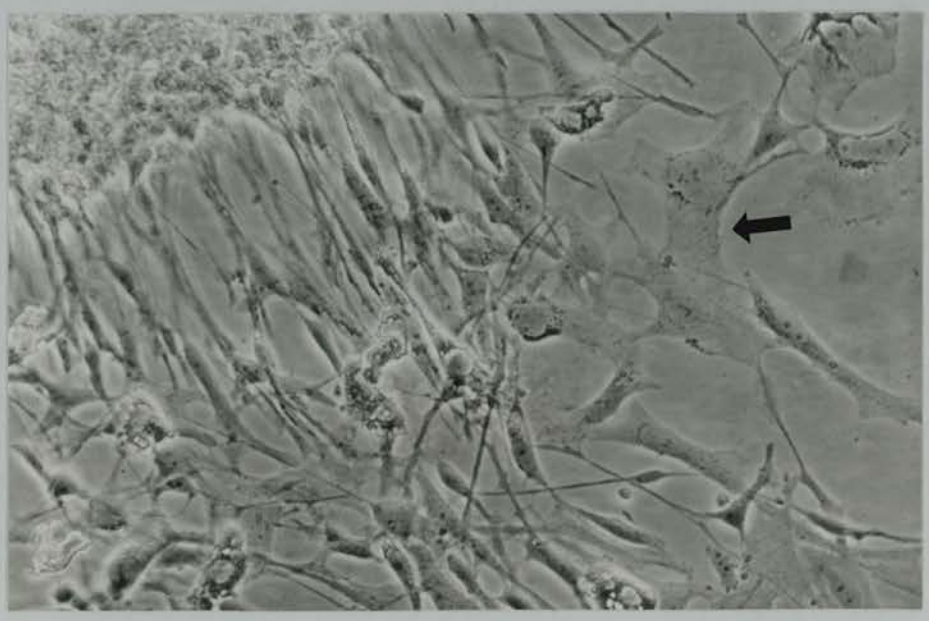
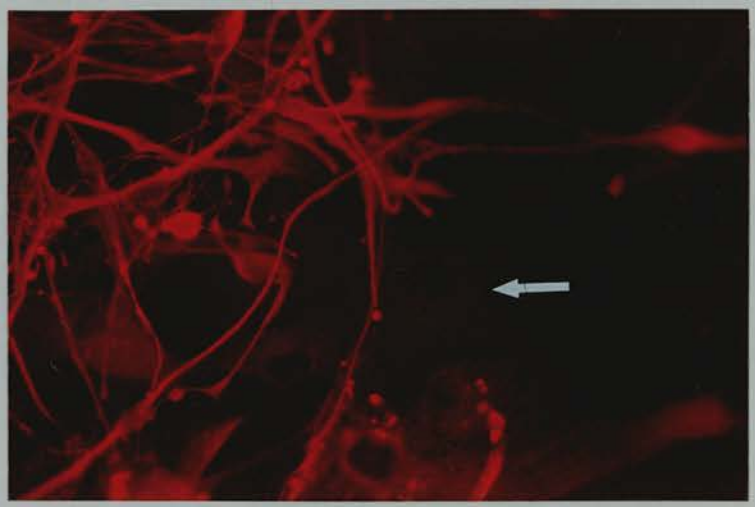
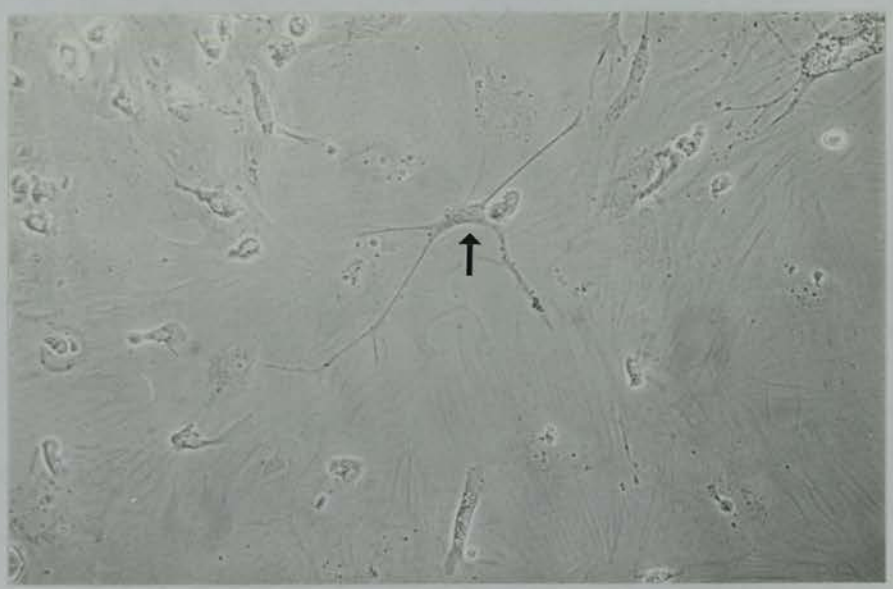


Fig.2.20 In contrast to the cells that form the bulk of the explant (at the left) the FA cells which can just be discerned at the right (arrow) do not express GFAP. High grade astrocytoma, 20 days in vitro, polyclonal anti-GFAP, x260

Fig.2.21 As FA cells come to dominate a culture so the PF cells diminish. A single PF cell (arrow) remains on the surface of underlying FA cells. The denser granular cells are degenerating PF cells. High grade astrocytoma, 23 days in vitro, phase contrast, x132

Fig.2.22 Single GFAP+ (orange) PF cells survive in relation to a proliferating mass of FN+ (green) FA cells. High grade astrocytoma, 36 days in vitro, polyclonal anti-GFAP, monoclonal anti-FN, x260





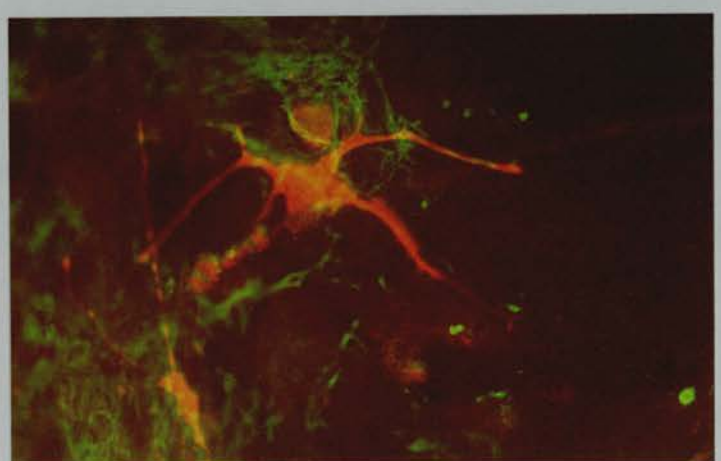
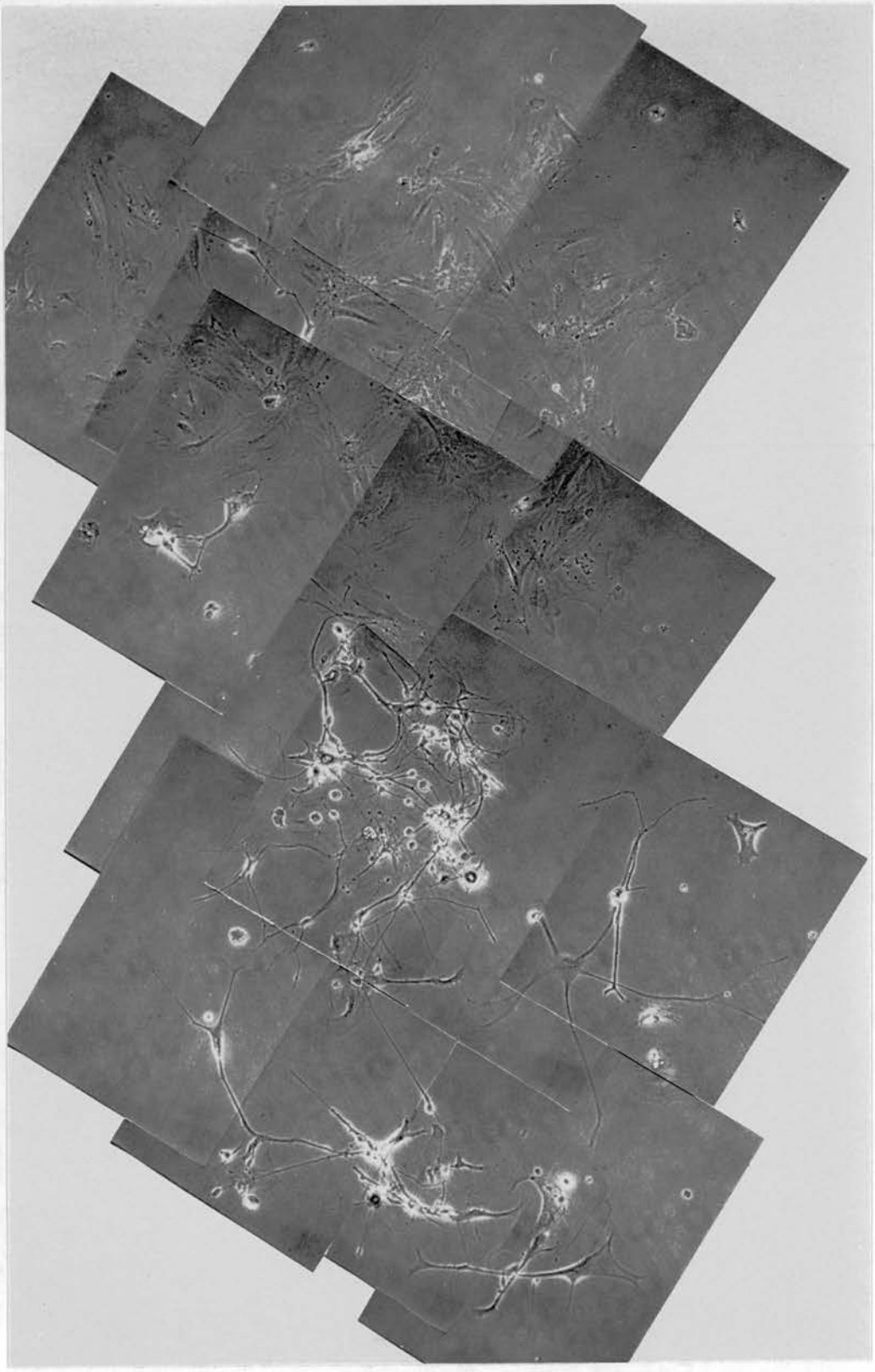


Fig.2.23 Composite photograph of a dispersed culture showing the simultaneous development of PF dominant (lower) and FA dominant (upper) areas. High grade astrocytoma, 21 days in vitro, phase contrast, x70

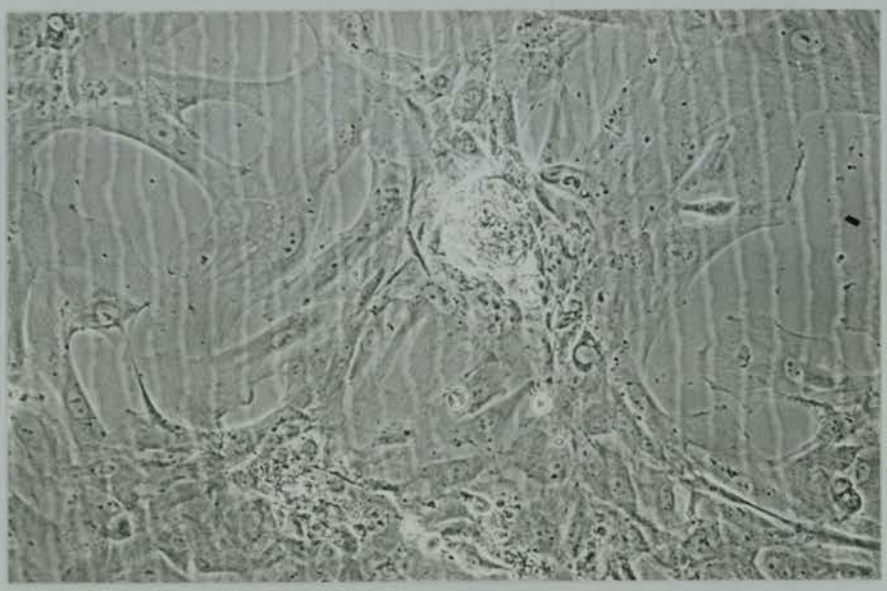


These FA cells formed a disorderly spreading population which only occasionally formed confluent monolayers, but instead formed cell clusters or aggregates piling up above the substratum and then forming sheets with continual focal heaping up of cells (fig.2.24) which were all FN+ (fig.2.25).
In dispersed cell preparations heaping disorderly growth of FN+ FA cells was seen as early as 9 days and in the absence of small explant-like fragments of tumour, by 21 days they had usually came to form the dominant population.

Small FA cells were rarely seen in low density but in cultures from five high grade astrocytomas, all older than 9 days, they formed more orderly growths resulting in a regular "cobblestone" pattern with some peripheral cellular irregularity but little overgrowing of one cell by another (fig.2.26). These cells invariably expressed plentiful surface FN but staining with Ulex europaeus lectin was always negative. In four such cultures (9 - 14 days in vitro) such sheets of small FA cells were negative for F8RAg. In four other cultures of similar age (8 - 14 days in vitro) from four different high grade tumours small FA cells formed small groups without either disorderly growth or sheet formation. In such circumstances the cells showed focally weak staining for F8RAg (fig.2.27) with strong surface FN staining.

Fig.2.24 FA cells showing aberrant growth, piling up to form heaps of cells. High grade astrocytoma, 27 days in vitro, phase contrast, x264

Fig.2.25 Extensive FN expression in a heap of FA cells. High grade astrocytoma, 31 days in vitro, monoclonal anti-FN, x260



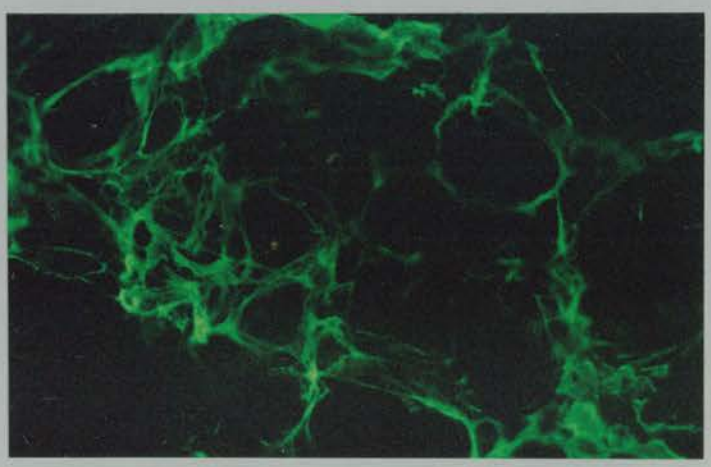
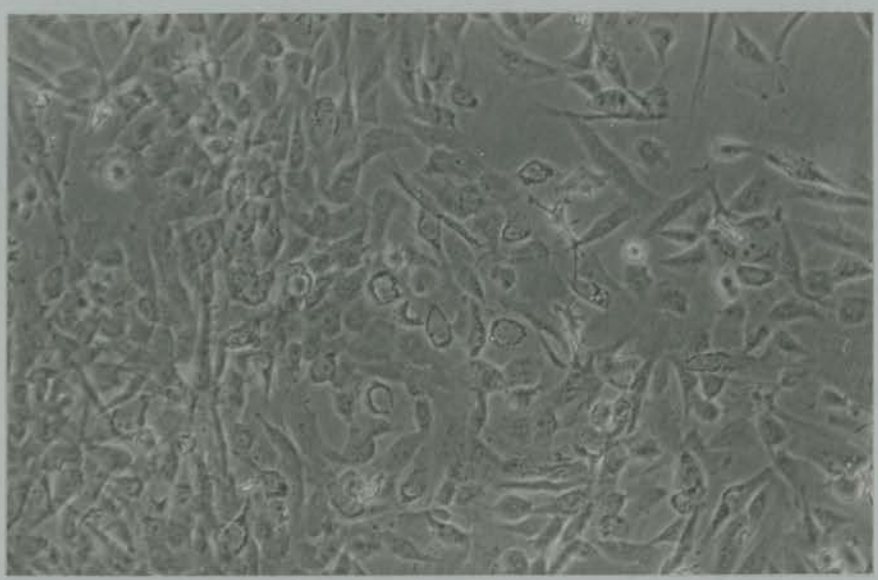
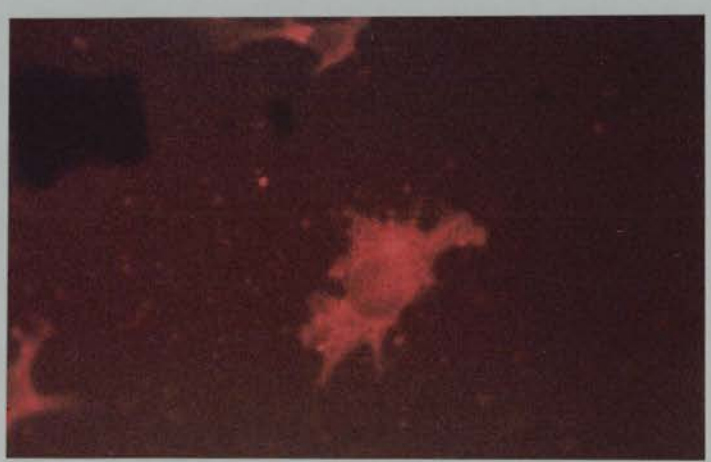


Fig.2.26 Small FA cells showing a relatively orderly growth pattern with minimal cell overlap. High grade astrocytoma, 22 days in vitro, phase contrast, x132

Fig.2.27 Single small FA cell in low density growth shows positive staining for Factor 8-Related Antigen (F8RAg). High grade astrocytoma, 8 days in vitro, polyclonal anti-F8Rag, x520





Three preparations, consisting mostly of large FA cells, were stained with Ki67. In two preparations at 16 days 25% of single cells, between 10% and 50% of cells in heaps and 3.7% of cells in sheets showed positive nuclear staining. In one preparation at 21 days 100% of cells at the edge of a disorderly sheet of cells showed Ki67 nuclear staining.

Although it was difficult, or impossible, to distinguish morphologically between the single interstitial FA cells in explants and the proliferating cells that were associated with PF cell loss they could be clearly separated by their immunohistochemical profiles. In contrast to the GFAP+ interstitial cells the proliferating, heaping, cells were uniformly strongly positive for surface FN and cytoplasmic vimentin, but always GFAP-.

This disorderly pattern of growth, apparently resulting from loss of contact inhibition, was seen in at least one culture in 24 out of 51 malignant astrocytomas and in 3 out of 6 low grade astrocytomas. The earliest time at which it was seen was after 6 days in-vitro but in one case did not appear until after 47 days.

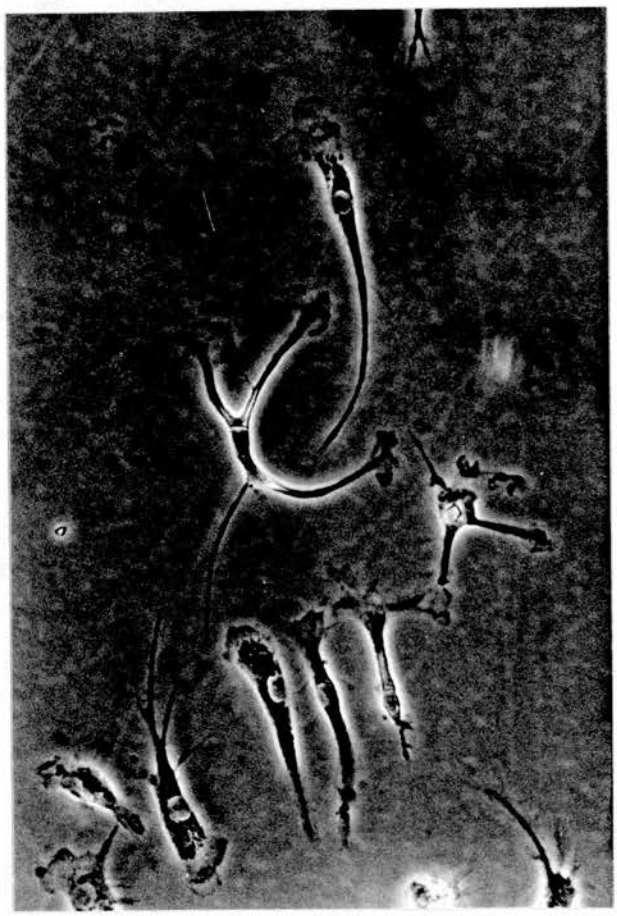
For the high grade tumours, when account was taken of the type of culture, striking differences were apparent. Explant cultures from 20% (9 out of 45) of tumours gave rise to abnormal FA cells compared to 57% of tumours (17

out of 30) when dispersed culture was used. The 37% difference was statistically significant (95% Confidence Limits 15%, 58%; p=0.0025, X^2 test). The time taken to develop such a pattern also varied, with median times of 17 days for dispersed cultures (range 9-27) and 21 days for explants (range 6-47). The numbers of low grade tumours examined was too small to allow comparable analysis but FA cells were seen after 30 days in one explant (1 out of 4 tumours), and after 13 and 15 days in two dispersed cultures (2 out of 3 tumours) (p>0.05, Fisher's Exact test).

c) Intermediate cells

These were of a variable morphology, usually with a polar elongation, a generally flattened adherent cell body and varying degrees of short process-formation (fig.2.28). They were usually found in low density, often in cultures from which few cells grew, or as a residual population in deteriorating low density cultures. They were not seen in higher densities nor as a growing population and were interpreted as an expression of motility in a variety of cells rather than as a defined cell population. This interpretation was borne out by the finding that there was no consistency in their staining reactions. Some were FN+/Vimentin+ but GFAP- whereas others (sometimes in the same specimen) were GFAP+/FN-.

Fig.2.28 Cells with intermediate morphology showing a range of shapes. Their refractile appearance is characteristic of cells in a deteriorating culture. High grade astrocytoma, 6 days in vitro, phase contrast, x132



Comparison of tumour types

The qualitative similarities of the growths from high and low grade astrocytic tumours has already been referred to, as has the similar frequency of development of FA cells. The effect of culture type on the pattern of growth of FA cells has already been described and a similar influence of culture type was apparent when the growth of cells whose processes formed complex networks, which analysis had shown to be consistently GFAP+, was considered. Such growth was seen from explants in 87% (39 out of 45) high grade tumours and in 100 % (4 out of 4) low grade astrocytomas and in dispersed cell cultures from 47% (14 out of 30) high grade tumours and in 100% (3 out of 3) low grade astrocytomas. None of these differences between tumour grades were statistically significant ($p>0.25$, X^2 test).

Effects of Passage

The effects of passage were studied in a total of 15 tumours (13 high grade astrocytomas, the subependymal astrocytoma and one ganglioglioma). Up to 10 serial passages were carried out (histochemistry was available up to the 7th), the maximum period in vitro being 100 days in the case of one high grade astrocytoma. As before the description that follows relates to the high grade tumours and separate consideration will be given to the subependymal astrocytoma and the ganglioglioma.

a) Residual cells

Trypsin/EDTA treatment resulted in extensive loss of PF cells and partial retraction of the cytoplasm of FA cells (figs.2.29a & 2.29b). Within 18 hours the latter cells reverted to their original flattened shape (fig.2.29c), began to proliferate and formed "nodes" (fig.2.30a) as seen in untreated areas of FA cell predominance and in the cells transferred by the passage process (fig.2.30b). This proliferation only occurred in areas where the cells were initially present in moderate density and was not seen to occur among single cells in low density. In the few areas where PF cells survived, the initial growth patterns were those of the original culture with the admixtures described.

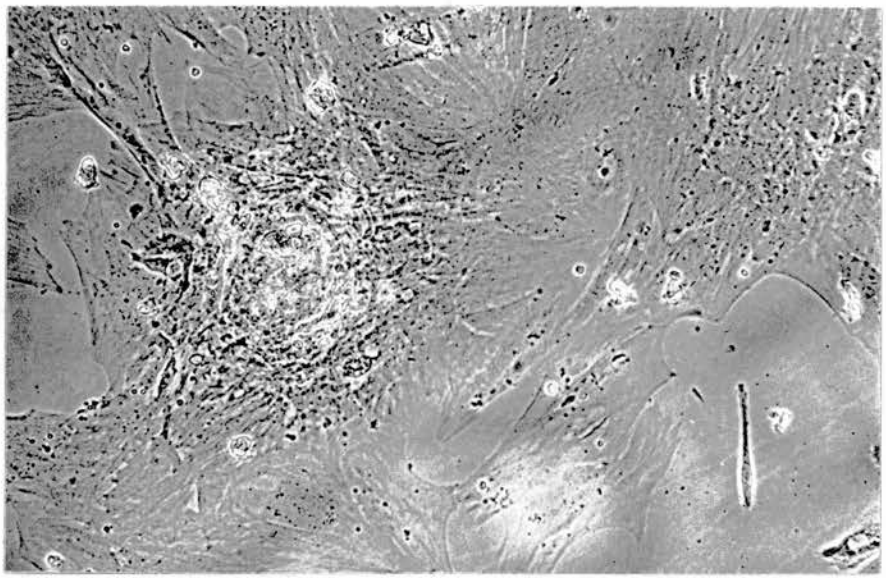
In three residual cultures studied 24 to 27 days after a single treatment with trypsin/EDTA (after a total of 48, 52 and 56 days in vitro) the cells were in one case entirely, and in two cases almost entirely, FN+/GFAP-/Desmin-/Vimentin+. Where other cell types were seen they consisted of a few residual GFAP+/FN- PF cells (24 days after treatment) and scattered single GFAP-/Desmin+ FA cells.

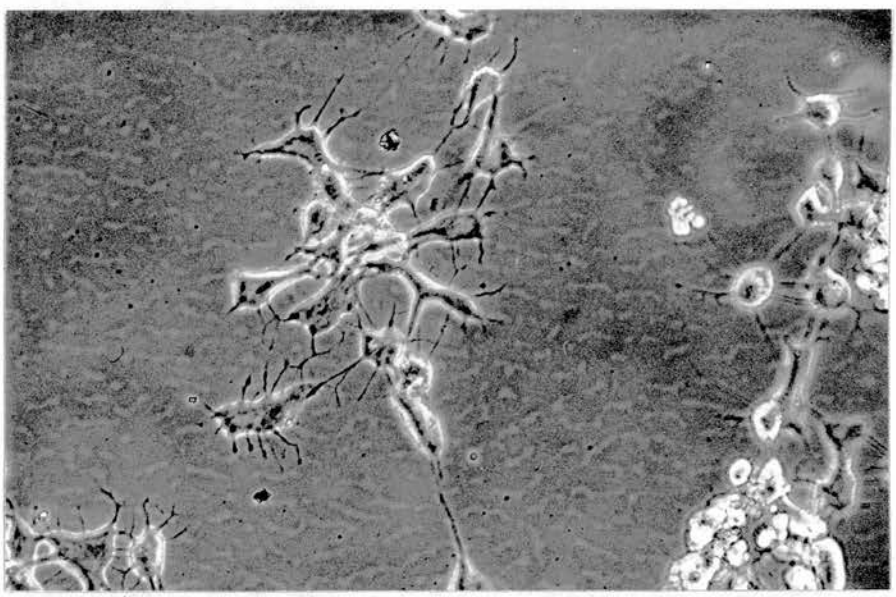
Fig.2.29 The effects of trypsin/EDTA treatment on FA cells from a High grade astrocytoma. All phase contrast x132

a. Disorderly heaps of FA cells prior to treatment. 26 days in vitro.

b. 30 minutes after treatment residual FA cells show retraction of their cytoplasm and rounding of the cell body.

c. After 24 hours the cells have regained their flattened appearance.





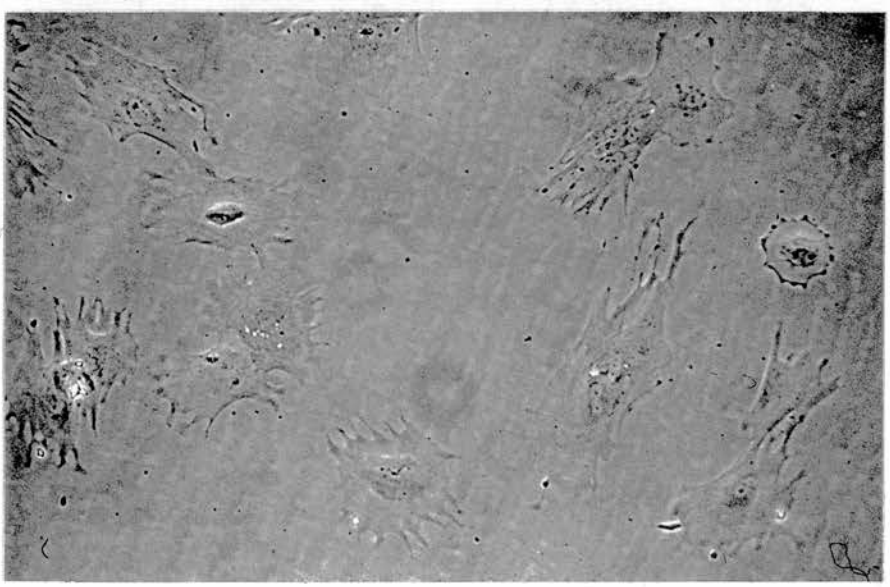
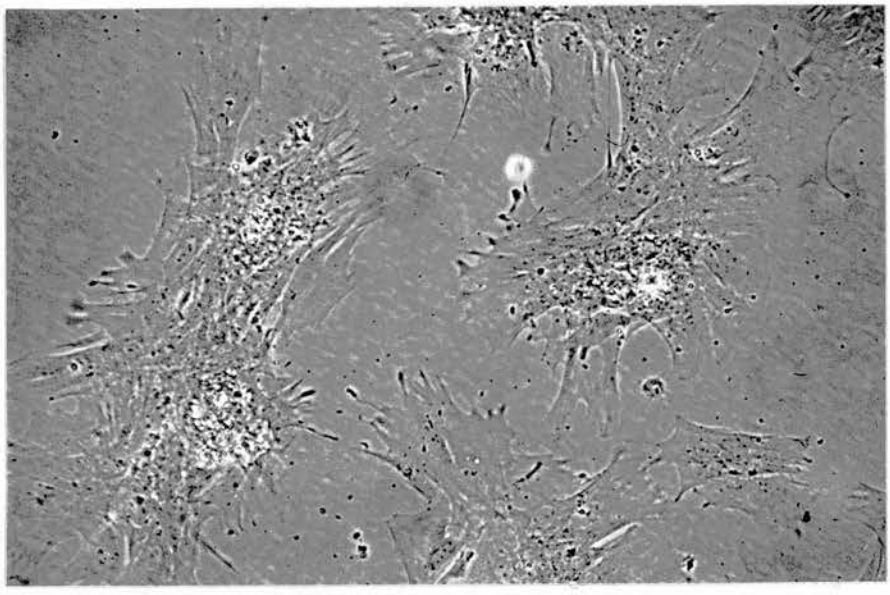
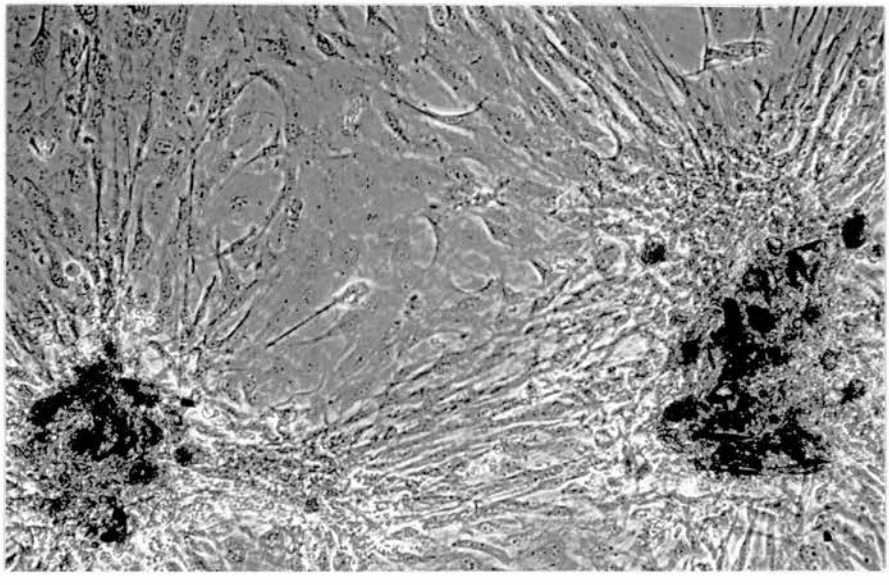


Fig.2.30. The effects of trypsin/EDTA treatment on FA cells from a High grade astrocytoma. All phase contrast x132

a. After 36 hours a disorderly proliferation is apparent.

b. Passaged cells 10 days after transfer show the disorderly heaping growth characteristic of FA cells.





To test whether the apparent enhancement of FA cell growth was due to some unexpected effect of the technique being used, a few cultures were treated with collagenase as in the initial cell dispersal. Although this was far less selective in its effects, both cell types being equally likely to be lost from the original culture, where medium to high densities of FA cells resulted from the elimination of PF cells the same enhanced pattern of FA cell proliferation was generally seen.

b) Passaged cells

Study of the cells transferred following treatment with trypsin/EDTA showed fairly consistent patterns of growth. It soon became apparent, however, that the morphology of the cells was not a reliable predictor of their histochemical reactions unless complex networks of cytoplasmic processes were formed, which invariably identified the cells as glial.

Although in three cases GFAP+ cells (both PF and FA in morphology) were still seen at 5, 7 and 11 days after passage, longer periods were associated with increasing dominance by FN+ cells with disordered growth and by 27 days after passage no GFAP+ cells were seen.
One exception to this was a culture that had been passaged 29 days previously and after 14 days had been treated again with trypsin. Although the residual cells 15 days after the second treatment were predominantly FN+/GFAP- a

few GFAP+ cells were seen which showed spotty deposits of surface FN.

After a second passage cultures maintained from 7 to 35 days all showed FN+/Vimentin+/GFAP- cells. As before these cells commonly showed the heaping growth pattern described above. In these and all subsequent passaged cultures no GFAP+ cells were seen.

In one case cells from passage number 3 were snap frozen in liquid nitrogen and recovered after 28 days storage. These too were all FN+/Vimentin+/GFAP- when studied after a further 14 days in culture.

Subependymal giant cell astrocytoma and Ganglioglioma

These two tumours differed from the majority of astrocytic tumours already described in that GFAP+ cells showed an unusual degree of persistence. Some of these GFAP+ cells were also unusual in showing degrees of coexpression of FN, although never in quantities seen in GFAP- FA cells.

1) Subependymal giant cell astrocytoma

Sections from the original tumour (fig.2.31) showed that the giant cell elements were predominantly positive for vimentin but not GFAP, while the latter was more commonly found in the smaller, more typically astrocytic cells with which the giant cells were mixed.

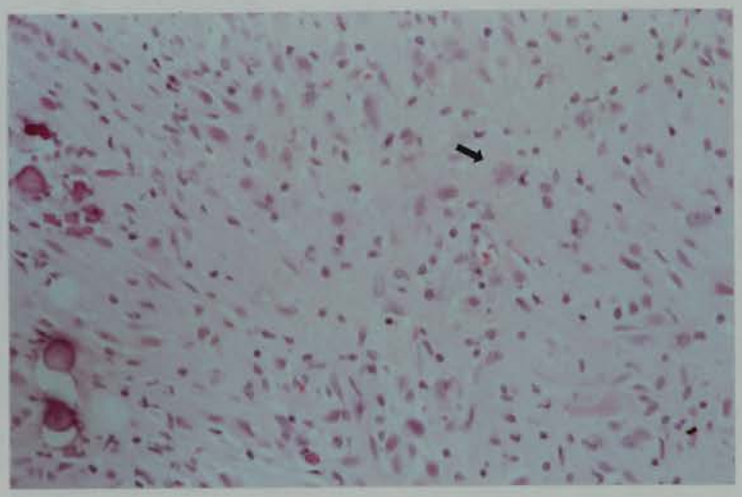
The general pattern of growth was similar to that seen in other gliomas with small fragments or larger explants forming complex networks from which, in time, flattened cells grew. These were frequently small and showed a far less disturbed growth pattern than that shown by the larger FA cells grown from other tumours and by 10 days they had formed endothelial-like clusters (fig.2.32). The PF cells showed considerable variation in size (fig.2.32) and were not invariably GFAP+, a number containing vimentin alone (fig.2.33). The endothelial-like cells showed much surface FN but this was not a feature of the PF cells.

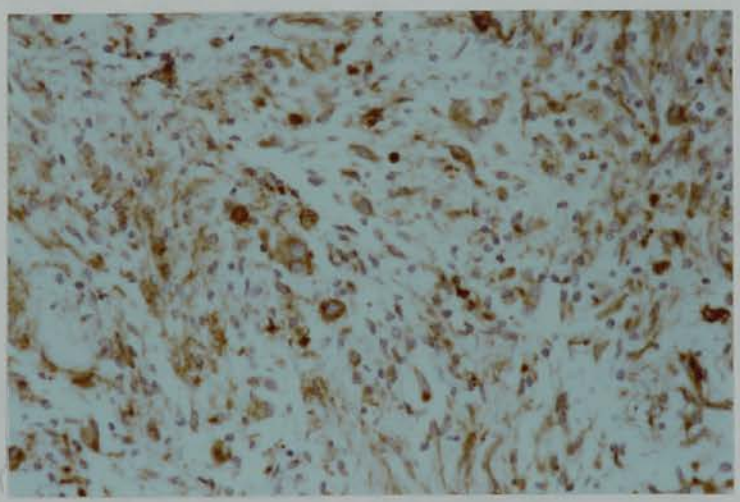
Fig.2.31 Supependymal giant cell astrocytoma.

a. Giant cells (arrow) contrast with background of smaller cells. Paraffin section, H&E, x260

b. Vimentin is present in both large and small cells. Paraffin section, monoclonal anti-vimentin, peroxidase, x260

c. GFAP is essentially restricted to the smaller cells, leaving the larger ones unstained. Paraffin section, polyclonal anti-GFAP, peroxidase, x260





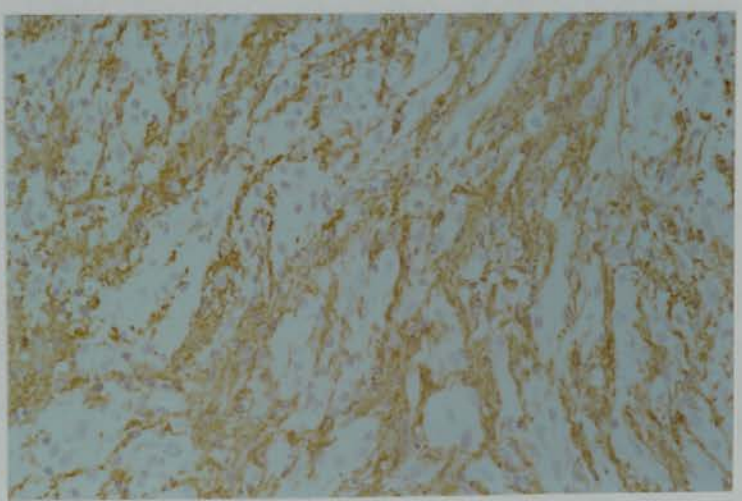
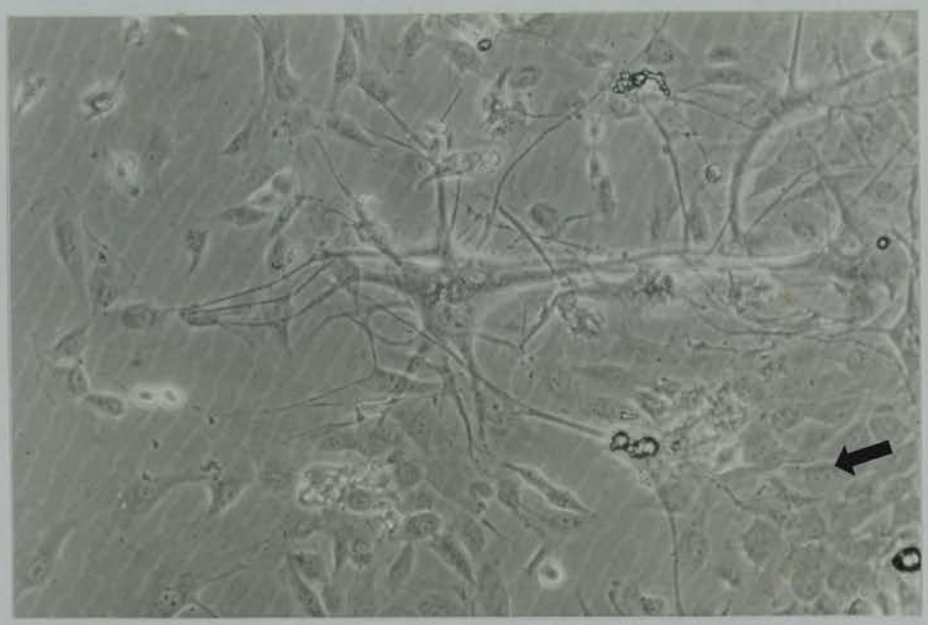


Fig.2.32 At the edge of an explant culture large (centre), and variable, PF cells are mixed with smaller endothelial-like cells (arrow). Supependymal giant cell astrocytoma, 15 days in vitro, phase contrast, x132

Fig.2.33 PF cell (left) and adjacent large FA cell (right) both show uniform staining for vimentin. Supependymal giant cell astrocytoma, 30 days in vitro, monoclonal anti-vimentin x260



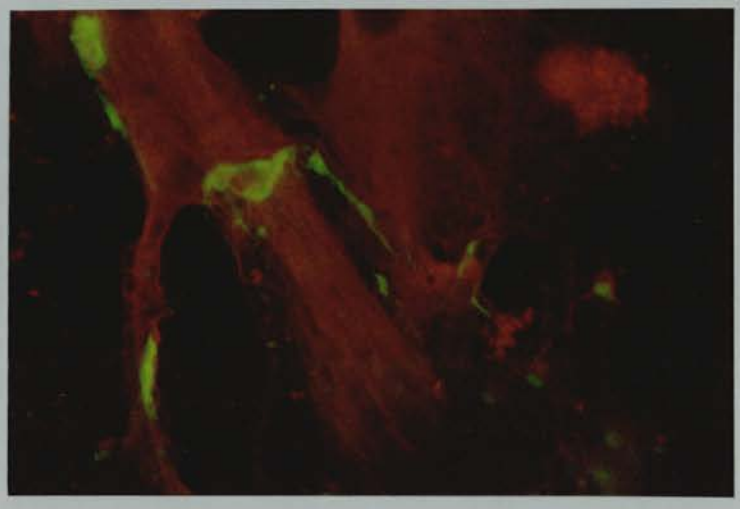
Some larger FA cells were seen and by 30 days some abnormal heaping of these cells was seen. At this stage some large FA cells showed strong vimentin staining and weak GFAP staining, while others showed strong vimentin staining, no GFAP staining and strong FN staining. PF cells, both singly and in small explant-like fragments, showed strong vimentin staining, usually (but not invariably) no GFAP and varying amounts of FN, although this was always focally, rather than uniformly, distributed over the cell surface.

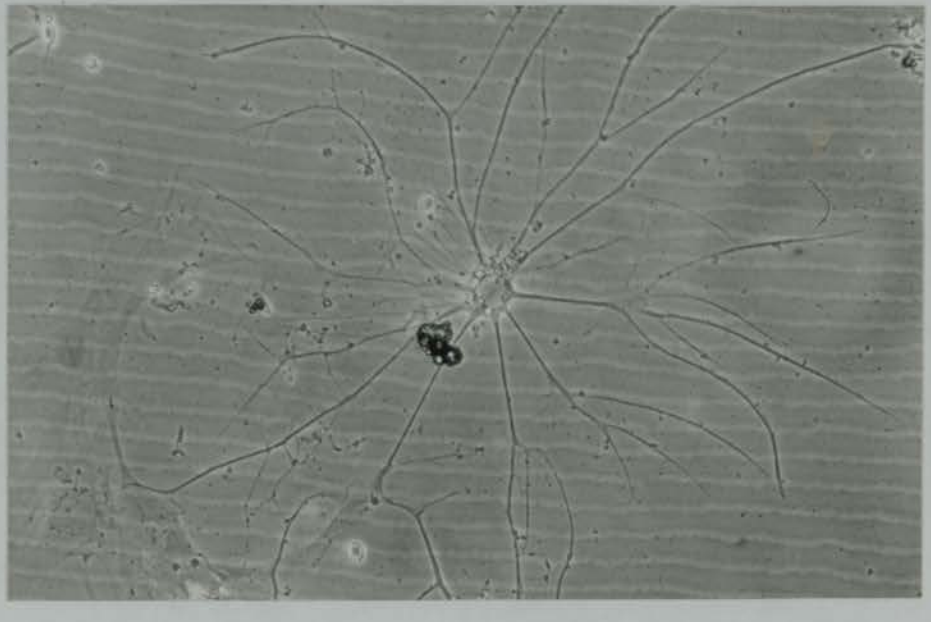
In a culture passaged after 10 days and observed after a further 31 days the mixture of cells described above persisted; some of the PF cells showed GFAP positivity and surface FN although the latter was never in the amounts seen on GFAP- heap-forming cells, and appeared to have been passively transferred from adjacent FN+ cells (fig.2.34).

After a second passage FN+/Vimentin+/GFAP- FA cells predominated and although they showed an irregular heaping growth pattern they were often smaller than the cells seen in cultures from other astrocytomas. Numbers of PF cells persisted (fig.2.35) and although they were mostly Vimentin+/GFAP-, some of them, together with some large FA cells, contained GFAP and bore varying amounts of surface FN.

Fig.2.34 Irregular strands of FN (green) adhere to GFAP+ (orange) cells but do not form a uniform surface layer. Supependymal giant cell astrocytoma, 49 days in vitro, second passage, polyclonal anti-GFAP, monoclonal anti-FN, x520

Fig.2.35 Large, complex, PF cell in first passage culture. Supependymal giant cell astrocytoma, 50 days in vitro, 7 days post-passage, phase contrast, x132





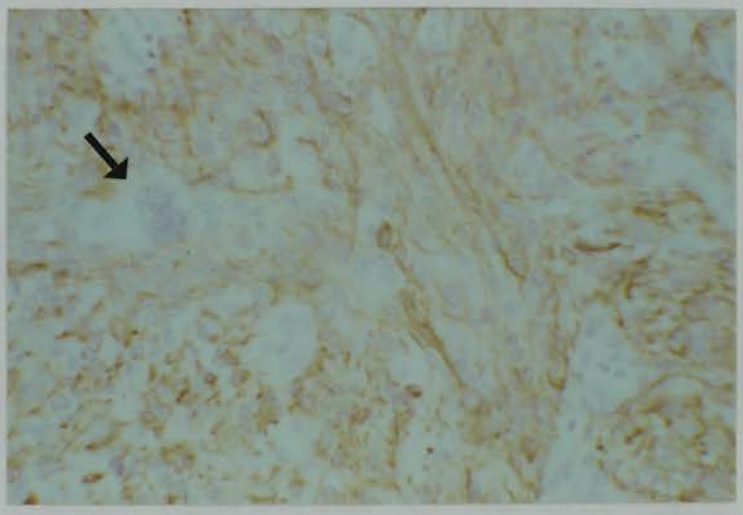
2) Ganglioglioma

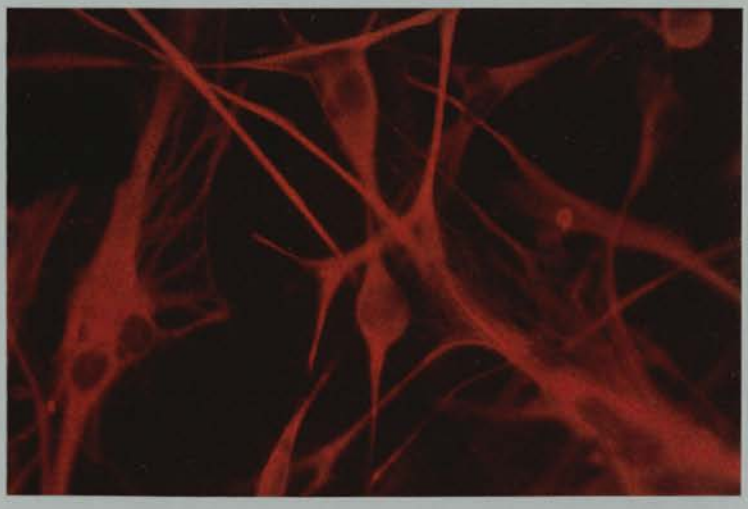
The original tumour showed a background of well differentiated fibrillary astrocytes with scattered abnormal ganglion cells, some of which were binucleate. The astrocytic elements were strongly GFAP+ contrasting with the negative neuronal elements (fig.2.36).
Primary cultures showed a predominance of GFAP+ PF cells in explant cultures and dispersed cell preparations with few FN+ FA cells (fig.2.37). After passage the numbers of FN+ cells increased although a disorderly growth pattern was not noted until after the fourth passage (27 days in culture). GFAP+ cells were still evident 7 days after the first passage (17 days in culture) and 17 days after the fourth passage (34 days in culture) (fig.2.38). In both these circumstances there were small patches of FN discernible on the cell surfaces, some of which appeared to have resulted from extension from strongly FN+ FA cells suggesting cell surface transfer of FN.

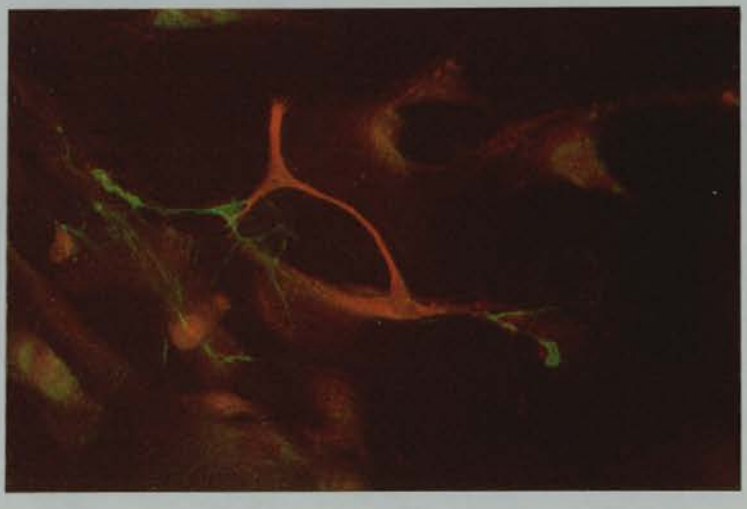
Fig.2.36 Ganglioglioma showing strongly stained glial elements and unstained large neuronal cells (arrow). Paraffin section, polyclonal anti GFAP, peroxidase, x270

Fig.2.37 Glial elements, with both PF and FA morphology, stain strongly for GFAP. Ganglioglioma, 13 days in vitro, polyclonal anti-GFAP, x260

Fig.2.38 GFAP+ cells (orange) persist after fourth passage admixed with FN+ cells (green). Ganglioglioma, 34 days in vitro, polyclonal anti-GFAP, monoclonal anti-FN, x260.







The properties of PF cells and the two types of FA cells can be summarised as follows:

PF cells

-consistently express GFAP and frequently express vimentin
-usually lack surface fibronectin
-can develop from explant or dispersed cell cultures but are more likely to persist in the former
-are usually lost with sequential passage
-persist in ganglioglioma and SEGCA cultures
-do not show overtly transformed properties

FA cells (i)

-properties as PF cells differing only in morphology

FA cells (ii)

-consistently express surface fibronectin and vimentin
-consistently lack GFAP
-can develop from explant or dispersed cell cultures but develop with significantly greater frequency and generally more rapidly from the latter
-become dominant population following sequential passage
-growth pattern suggests a transformed nature

DISCUSSION

The observation that human malignant glial tumours in culture give rise to a variety of morphologically distinct cell types has been made in a number of studies (Frame et al, 1984; Franks et al, 1986; Jacobsen et al, 1987; Kennedy et al, 1987; Lolait et al, 1983; Lumsden, 1971; Manoury, 1977; McKeever et al, 1987; Paetau, 1988; Ponten et al, 1978; Rutka et al, 1987a). Interpretations of the nature of the cells have varied, and although most workers have identified glial elements with varying degrees of certainty (depending on whether or not immunohistochemical confirmation was carried out) some have also argued that non-glial elements may be developing in-vitro (Franks et al, 1986; Jacobsen et al, 1987; Manoury, 1977; McKeever et al, 1987; Rutka et al, 1986a; Rutka et al, 1987a).

The ability to identify GFAP immunohistochemically in tissue sections led to the recognition that it was present in neoplastic cells in the majority of astrocytic tumours (Deck et al, 1978; Eng et al, 1978). Not all tumour cells expressed GFAP and anaplastic tumours were more likely to contain such negative cells (Eng et al, 1978) but the relationship between intermediate filament expression and cellular differentiation was far from clear. Developmental studies of glial cells in vivo had suggested that GFAP replaced vimentin as glial cells matured (Bignami et al, 1985) but in vitro work had shown that GFAP could be

induced by a variety of external factors including hydrocortisone, prostaglandins, Fibroblast Growth Factor (Morrison et al, 1985) and extracellular matrix (Rutka et al, 1987). GFAP expression in neoplastic cells was found to be increased when they were in close relation to mesenchymal tissue, either when invading the meninges (Herpers et al, 1984) or in extracranial locations such as liver (Kawasaki et al, 1987). This led to the suggestion that GFAP was of importance in both cytoskeletal construction and the subsequent maintenance of the "mature" or process-forming phenotype, whether induced as a part of the process of normal development or in response to external factors acting on neoplastic cells (Eng et al, 1982; Morrison et al, 1985). The fact that staining for GFAP in neoplastic astrocytes tends to be stronger in cell processes (Duffy et al, 1982; Herpers et al, 1986) gives some credence to this interpretation. Related to this idea was the suggestion that paucity of GFAP in some tumour cells was responsible for the characteristic pleomorphism of malignant gliomas (Duffy et al, 1980).

Although all mature non-neoplastic astrocytes were found to express GFAP only some expressed vimentin (Lazarides, 1982). The significance of the pattern of expression of these two intermediate filaments is far from clear but it has been suggested that different cellular functions may impose different structural requirements which are met, in part, by the assembly of intermediate filaments appropriate to the cell's needs. As an example the

expression of vimentin by astrocytes involved in the healing of an experimental injury was attributed to a proliferative state and a possible need for motility rather than to cellular immaturity (Schiffer et al, 1986a). The direct observation in vitro of the relationship between motility and GFAP expression supports such a view (Duffy et al, 1982); GFAP was found to correlate inversely with the degree of motility of human astrocytoma cells and was postulated to be directly involved in process extension but to inhibit cell movement. Others have considered the possibilities that the patterns of expression may have no functional significance or may be related to some undetermined regulatory function (Sharp et al, 1982).

The question of the relationship between GFAP and vimentin has been addressed by a number of studies of human and animal astrocytes, both normal and neoplastic. In human astrocytic tumours vimentin and GFAP are usually co-expressed although not every cell contains both filament types and there does not appear to be any clear correlation between the degree of vimentin expression and tumour grade (Herpers et al, 1986; Reifenberger et al, 1987; Schiffer et al, 1986; Yung et al, 1985). The latter point contrasts with the situation observed in a chemically-induced animal glioma where increasing cellular anaplasia was associated with increased numbers of vimentin-containing cells (Yoshimine et al, 1988).

The co-expression of two intermediate filaments, which has also been observed in vitro in the cells of an established human glioma cell line (Sharp et al, 1982), has been attributed to copolymerisation with coassembly of filaments from a pool of common precursor fragments (Sharp et al, 1982; Wang et al, 1984).

Any immunohistochemical analysis of the pattern of intermediate filament expression must recognise that variations in reported patterns may reflect differences in the affinities and specificities of the antibodies used. This is well illustrated in the case of desmin where studies of astrocytes variously reported that it was present (Dahl et al, 1982, 1986) or absent (Debus et al, 1983; Raemaekers et al, 1983). These findings were reconciled by the suggestion that desmins from different sites or cell types may differ antigenically, and antibodies raised against one type might not necessarily react with another type in a different location (Dahl et al, 1986).

The expression of GFAP, vimentin and occasionally desmin, but not fibronectin, by the majority of PF cells and by the FA cells in interstitial locations within networks formed by such cells is sufficient to identify them both as being glial in nature.

In the cultures studied with Ki67 antibody these cells are

seen to be actively dividing although the percentage of cells thus engaged was generally small. Although small spots of fibronectin were seen on occasional glial cells dense surface and interstitial deposits were never a feature. It is of interest, however, that in both the subependymal giant cell astrocytoma and the ganglioglioma, fibronectin was more prominent on glial cells (although never as a dense surface network) and that in these tumours glial cells apparently persisted longer than was usual in malignant astroglial tumours.

A dense surface and interstitial pattern of fibronectin deposition characterised the population of flattened adherent cells with a heaping growth pattern. These cells always expressed vimentin, but never GFAP or desmin. These cells, as simple morphological observations of their growth would suggest, were found to have a far higher proportion of cells in a proliferative state (as identified by Ki67 staining), especially in the areas of abnormal heaping growth. Although the pattern of fibronectin staining indicated both surface and interstitial deposition of the type described in a number of studies of normal fibroblasts (Ruoslahti et al, 1981; Bitterman et al, 1983; Vaheri et al, 1976; Lim et al, 1982; Trejdosiewicz et al, 1985; Fromme et al, 1982) the failure to form monolayers and the frequency with which growth formed local heaps were quite unlike the pattern typical of normal fibroblasts in culture (Vasiliev et al,

1981). None of these cells expressed either of the two endothelial markers used and they appeared to differ from the smaller cells which were occasionally observed to form small clusters with inconstant staining for F8RAg, or larger regular cobblestone like sheets without F8RAg reaction. These latter cells would appear to be endothelial despite the absence of UEl reaction. The apparent loss of F8RAg in later cultures suggests that absence of endothelial markers may not be a valid criterion for excluding an origin of the larger FA cells from endothelium; this is in accord with deBault's finding that F8RAg is lost early in cultures of known endothelial origin (deBault, 1982). That readily identifiable endothelial-like elements survive and grow from human gliomas seems likely, but the relative infrequency of this suggests that their growth (in their ordinary state at least) is not favoured by the conditions of culture used in the present studies.

In the work presented in this thesis culture of a variety of astrocytic tumours has shown remarkably consistent patterns of growth. There is a clear contrast between cultures in which explants are established and those in which the component cells of the tumour are initially separated by the action of collagenase. In the former astrocytic elements persist in a coherent network which shows progressive peripheral extension, although with time focal proliferations of FN+ cells develop and either

overwhelm the explant, or inhibit further extension. In dispersed cell preparations low densities of glial cells fare badly, and rarely form thriving cultures, whereas these conditions favour the emergence of FN+ cells even though parallel explant cultures from the same tumour show that only very small numbers are present initially.

The growth pattern of the FN+ cells is disorderly, they appear to demonstrate loss of contact inhibition, and they are capable of surviving up to 10 passages. This contrasts with the glial cells (identified by their GFAP content) which in their typical process-forming state rarely show cell crowding and instead preserve considerable cell-cell distances although interconnected by cell processes. Those glial elements which show less process formation remain dispersed through the interstices of explants and were not seen to form cell masses comparable with those of the fibronectin-bearing cells.

The present studies show that the manipulations associated with passage have a profound effect on the populations of cells available for study; they serve to destroy the microenvironment in which cells are developing and thus alter the conditions in ways that may favour the differential survival and growth of a single cell type. Thus passage enhances the emergence of FN+ cells to the detriment of glial elements that will survive for far longer periods if left undisturbed. This suggests that the

glial cells depend for their survival on environmental conditions which are disrupted by passage and possibly compromised by techniques that utilise disruption of tumour tissue prior to culture.

Three major questions need to be addressed:

i) Why do the GFAP+ glial cells, in high density, maintain a relatively orderly, complex, network and yet, in lower densities, fail to survive (with few exceptions) in prolonged culture?

ii) What underlies the apparently transformed behaviour of the FN+ cells?

iii) What is the origin of the FN+ cells?

Behaviour of glial cells in culture.

Studies of embryonic cell populations in culture (Steinberg, 1970; Townes et al, 1955) have given some indications of how cells of differing type interact. Cell motility and differential adhesion will allow cells of similar types to combine to form tissues in ways which can be modulated by those surface properties that determine such adhesion. The adhesion of like cells is seen in the formation of tumour cell spheroids (De Ridder et al, 1987; Mareel, 1983) and in embryonic tissues; in the latter changes in adhesion preference with time (one element of embryonic differentiation) will alter the pattern of adhesion allowing the progressive restructuring of tissues which is necessary for organogenesis. Such a process is considered to underly the "sorting out" which is seen among mixed embryonic cell populations (Steinberg, 1970), and may also be operating in the mixed cell populations which result from primary dispersion of tissue from a glioma.

Cell contacts have been shown, in some cases, to be via specialised low resistance junctions (Furshplan et al, 1968) which may allow ionic transfer and thus effect communication. It has been suggested that ionic coupling of cells through such junctions allows the decision to be made whether cells will bind or not. The active extension of processes by glial cells and the making of contact with other glial cells and FA cells could provide the means for

such communication. There is certainly evidence that aggregations of glial cells, both normal and neoplastic, spread and become polarised with respect to their substrate more rapidly than do single cells (Forsby et al, 1985). The persistence of density-dependence, as far as growth is concerned, among cells derived from a number of glial tumours (Westermark, 1973) and the maintenance of a relatively orderly network in explants may reflect the importance of cell contacts both in maintaining growth, and controlling it in a way that results in a form of contact inhibition. Such contacts would be extremely susceptible to physical disruption and manipulations such as passage would be expected to reduce the chances of re-establishing communication and so reduce survival, or at least active growth. The process of passage might be more likely to disrupt such an active mechanism of cell communication than one dependent on cell binding based on fibronectin and transmembrane modulation via actin, a mechanism conceivably utilised by FN+ FA cells. As a result passage would be expected to favour the survival of FA cells rather than PF cells.

The fact that glial elements survived longer in cultures from two extremely low grade tumours (the subependymal giant cell astrocytoma and the ganglioglioma) would require confirmation with larger numbers but it does suggest that the degree of malignancy of the original tumour is not a good predictor of survival in the type of

culture system used in the experiments described above. This is of interest in the light of the observation that cell lines have never been developed from well differentiated gliomas (Collins et al, 1980; Ponten et al, 1973; Onda et al, 1988) and touches on the question of what range of transformed properties are actually possessed by the parenchymal cells of a glioma. Thus the failure of PF cells to survive on the surface of FA cells may be a "toxic" effect, may indicate the retention of a specific anchorage dependence normally only satisfied by the properties of the culture flask, or may result from an interference with the formation of cell-to-cell contacts required for glial cell survival. The fact that the glial components were influenced by FA cells cannot be taken to indicate that they are not neoplastic since some metastasising clones of HeLal cells have been found to be incapable of attaching to, or penetrating fibroblast monolayers, in apparent contrast to their in vivo properties (Benke et al, 1984).

FN+ cells appear more readily in cultures from dispersed tumour preparations, and only slowly from explants. It is possible that the structures in the original tumour from which the FN+ cells derive require to be disrupted before the cells can grow. Alternatively there may be an inhibitory influence of glial cells (which grow better in explants) on the growth of the FN+ cells. Gliomas are known to produce growth inhibiting factors, which act on T

lymphocytes and neuroblastoma cells (Fontana et al, 1985), and it is possible that factors may be produced in vitro that act on the progenitor cells of the FN+ population. The manipulations of passage could therefore be seen as reducing the glial population and thus minimising any inhibitory effect it might have on FN+ FA cells.

The nature of the interstitial GFAP+ cells in the interstices of the glial networks is not clear. They may be cells in the process of migration within the culture which have not formed contacts, or conceivably cells which are more transformed and do not need to form such contacts. In vitro studies of rat glioma cells have shown that FA-like cells can be induced by exposure to Nerve Growth Factor whereas Glial Maturation Factor enhanced process formation and the development of PF-like cells (Marushige et al, 1987) suggesting that the two cells types are interconvertible depending on environmental influences. The invariable presence of GFAP+ FA cells in high density PF growths also raises the possibility that the latter may derive some stimulus from the former and the cis-oncogene product Platelet Derived Growth Factor (PDGF), which is produced by some glioma-derived cells (Westermark et al, 1985), has been suggested as playing a role in such an interaction (Nister et al, 1986). The techniques used in the studies presented in this thesis did not allow any phenotypic distinction to be made between those glial cells that formed contacts in networks

and those that did not. Nevertheless there is considerable heterogeneity among cell lines derived from gliomas (Jacobsen et al, 1987; Bigner et al, 1981; Rutka et al, 1987a; Dewhurst et al 1987; Bullard et al 1981) including heterogeneous secretion of PDGF (Nister et al, 1985, 1986). Studies of the morphology of cloned cell lines producing PDGF or cells bearing PDGF receptors have suggested an inverse relationship between the two properties: high receptor levels characterise large stellate cells, and high PDGF production is a feature of smaller polyhedral cells (Nister et al, 1986). Similar studies in mixed culture would be of considerable interest and might shed further light on the interdependence of the two morphological variants of glial cell observed in explants in the present study.

The apparent paradox that cells derived from a tumour and considered to represent the parenchymal, that is the neoplastic, population do not show all the properties of transformed cells was discussed earlier (page 86 et seq). Whilst it is apparent that neoplastic transformation is associated with abnormal cell behaviour there is ample evidence that transformed cells still retain, to varying degrees, many normal properties (Vasiliev et al, 1981), including residual contact inhibition (Westermark, 1973). It has been shown that during transformation the ability of cells to induce angiogenesis is acquired before the ability to form a tumour (Ziche et al, 1982), and antigenic heterogeneity has been shown to exist between

primary tumours and their metastases (Enblad et al, 1985; Parmiani et al, 1985), suggesting that there may be considerable diversity among cells in a tumour in terms of the possession of transformed or neoplastic properties as well as in phenotype and genotype (Shapiro et al, 1985). The persistence of the property of density dependent growth may well be of importance in explaining the general absence of metastases from malignant glial tumours despite ultrastructural evidence that penetration of tumour blood-vessel walls by neoplastic glial cells does occur (Kung et al, 1969).

Behaviour of Fibronectin positive (FN+) cells

FN+FA cells are more likely to appear in early cultures derived from dispersed preparations than from explants although they will eventually appear in a considerable number of the latter if culture is continued for long enough. Although they appear to be inhibited by high density growths of PF cells, this can be overcome by artificially reducing PF cell density by passage. The question of the nature and origin of the FN+ FA cells must be addressed in view of the observation that they appear to increase in number in culture and yet may not be common in vivo (Schiffer et al, 1984; Chronwall et al, 1983; Kochi et al, 1983; Paetau et al, 1980a). On the basis of their morphology and non-confluent growth pattern they do do not appear to be fibroblasts, and yet they appear to be

antigenically distinct from the glial elements, confirming the mutual exclusivity of GFAP and FN expression reported by others (Paetau et al, 1980a; McKeever et al, 1987; Paetau, 1988). The non confluent growth pattern with "node" formation resembles the artificially induced cell islands grown from glioma derived cells (Collins et al, 1980) and suggests a transformed nature as well as an ability to adhere to each other in preference to the substrate. FA cells appear to correspond to the cells present in 80% of the gliomas studied by Kennedy and colleagues in a dispersed cell system (Kennedy et al, 1987). These bore surface FN which might be expected to enhance initial cell adhesion and thus survival, and similar cells have been reported to be derived from gliomas in a number of other studies (Franks et al, 1986; Lolait et al, 1983; McKeever et al, 1987; Paetau, 1988).

FN is known to play an important role in mediating the adhesion of cells to extracellular matrix (Yamada et al, 1978) and to each other (Saison et al, 1983) although the latter function is also mediated by other unidentified surface proteins. This process of cell to cell adhesion appears to be an active one requiring prolonged cell contact rather than a passive "gluing" phenomenon, implying that alterations in the cell surface need to occur. In addition to its binding properties FN appears to play a role in stimulating (Gullino, 1986; Rennard et al, 1981; Tsukamoto et al, 1981), facilitating (Schor et al,

1981) and directing cell migration, possibly by providing sites for preferential binding (Schor et al, 1983), or by acting as a "guidance trail" (McAuslan et al, 1980) in a way analogous to the extracellular mucins produced by slime moulds (White, 1981). In the latter role FN may be only one of a number of extracellular matrix components that are of importance in determining the growth of cells and the direction of cell movement. There is evidence that these substances are also required, in a permissive sense, if cells are to be responsive to external growth factors (Gospodarowicz et al, 1980), a fact that reinforces the importance of substrate adhesion in the proliferation of normal cells.

The observation that cell-associated FN was reduced with viral transformation (Adams et al, 1977) and the finding that FN will restore a normal appearance (Yamada et al, 1976), although not necessarily a normal growth rate (Mosher et al, 1981), to transformed cells would seem to argue against identification of the FN+ cells observed in this study as neoplastic. As mentioned earlier (page 31) the basis for FN loss in some transformed cells may be in their ability to retain FN on their surface rather than in the synthesis of the substance (Ruoslahti et al, 1981). Indeed the correlation between reduced surface FN expression and transformation is not an absolute one and tumourigenic cells been described that bear surface FN (Khan et al, 1979; Pearlstein et al, 1976). It has also

been postulated that point mutations in genes coding for FN structure may result in a functionally defective protein (Ruoslahti, 1984), a view supported by the known importance of FN structure in determining its cell-binding function (Ali et al, 1978; Grinnel et al, 1980). Discrepancies have also been observed between the in vivo and in-vitro expression of FN (Chen et al, 1979). While this suggests that tumour cells may express (or retain) surface FN in culture and may not do so when forming tumours in vivo, an alternative explanation is that such phenomena represent differences between clones arising within the same tumour (Jiwa et al, 1985).

The nature of the studies described in this thesis did not allow the assessment of other transformed properties, such as growth in soft agar, enhanced cloning ability or tumourigenicity, and, although survival for up to 10 passages was observed, continuously growing cell lines were not developed. Before coming to a final conclusion about the nature of these FN+ cells it is necessary to consider the possible identities of their cells of origin and possible sources within the tumours from which they are derived.

Possible origins of Fibronectin positive (FN+) cells

There are two main schools of thought concerning the origin (and thus the nature) of the FN+ cell.
Either i) the cell is genuinely neoplastic, is of glial origin, and has a phenotype indicative of a less differentiated state than those cells which express GFAP and not FN (Frame et al, 1984; Kennedy et al, 1987)
or ii) it is not of parenchymal origin but instead is derived from mesenchymal or endothelial elements in response to factors produced by the tumours (Franks et al, 1986; Jacobsen et al, 1987; McKeever et al, 1987; Manoury, 1977; Rutka et al, 1987a).

Is the FN+ cell glial? Evidence in favour of the view that the FA cell is neoplastic and glial includes an aberrant growth pattern, its lack of contact inhibition, its consistent derivation from glial tumours (if cell dispersal methods are used), the finding of chromosomal abnormalities in some cells (Kennedy et al, 1987) and an association with fibrinolytic activity (Frame et al, 1984; Freshney et al, 1985; Freshney et al, 1985; McLean et al, 1986).

The lack of glial intermediate filament expression by cells of putative glial origin is consistent with the observations made on cells from optic nerve of the developing, and adult, rat (Ffrench-Constant et al, 1986; Raff et al, 1983). These studies demonstrated that an

astroglial precursor cell may not express specific astroglial intermediate filaments, and observations on human astrocytic tumours have similarly demonstrated that cells may be present, although in varying numbers, that do not express histochemically detectable GFAP (Franks, 1988; Roessmann et al, 1983; Schiffer et al, 1984; Schiffer et al, 1986; Yung et al, 1985). This lack of expression does not appear to relate to tumour grade (Herpers et al, 1986) and GFAP expression may be affected by the local microenvironment of the neoplastic cells rather than be determined solely by the degree of cell differentiation (Herpers et al, 1984; Morrison et al, 1985; Rutka et al, 1987; Kawasaki et al, 1987).

If the FN+ cells are to be interpreted as being of glial origin they might be expected to have a phenotypic counterpart among normal glial cells. Early studies on cells derived from normal human brain yielded flattened polygonal cells with minor degrees of process formation which were accepted as glial without further characterisation (Ponten et al, 1969); these cells were subsequently found to express surface FN (Vaheri et al, 1976). Although these authors observed that cells derived from non-CNS tumours secreted FN and failed to retain it on their surface, FN-producing cells isolated in vitro from human gliomas were considered to be neoplastic counterparts of the normal "glial" cells which had already been described. Normal rat astrocytes, whose nature has

been confirmed by the presence of GFAP, have been shown to secrete FN up to nine days postnatal (Liesi et al, 1986). However in the majority of such cells the FN is in the form of punctate deposits (as seen in the few GFAP+ cells in this work which were also FN+) rather than the fibrillar matrix seen in fibroblasts (Price et al, 1985) and in the FA cells. In other words astrocyte FN is secreted but not readily incorporated into a matrix. Nevertheless results from a number of studies were considered to provide a measure of support for the view that GFAP-/FN+ cells were of glial nature.

Continuous cell lines derived from gliomas, and therefore assumed to be neoplastic, may not express GFAP, or may express FN (Bigner et al, 1981; Dewhurst et al, 1987; Jones et al, 1980; Jones et al, 1982); GFAP may be lost from cell lines with serial transfer (Jones et al, 1981); in-vitro transformation of glial cells may result in reduced GFAP expression (Laerum et al, 1985) or reduced inducibility of GFAP by exogenous maturation factors (Haaugen et al, 1981); GFAP+ cell lines may be induced to express FN if they are allowed to form tumours in athymic mice (Jones et al, 1982). These findings could, however, all be interpreted as the result of selective survival and growth enhancement of a population of non-glial cells under experimental conditions. Nevertheless the view was propounded that neoplastic glial cells need not express GFAP, and may, in appropriate circumstances form matriceal

rather than soluble FN resulting in the phenotype associated with FA morphology.

Steroids are known to be capable of inducing differentiation in neoplastic astroglial cells (Freshney, 1985; Freshney et al, 1986; McLean et al, 1986) and both dexamethasone and methyl prednisolone have been found to increase cellular GFAP in a human astrocytoma cell line (Pilkington et al, 1987). The latter group have also found that if cultures, in which GFAP+ cells are normally lost and replaced by FN+ cells, are treated with methyl prednisolone the GFAP+ cells persist for longer (Wright et al, 1987); these results were interpreted as indicating that the FN+ cells were being induced to differentiate to GFAP+ cells. Steroids are however known to increase FN expression in transformed cells (Furcht et al, 1979); the increased expression of GFAP, in the culture as a whole, could have been due to increased FN expression by glial cells which thus acquired a survival advantage in the passaging process by virtue of enhanced cell adhesion (Grinnel et al, 1980).

Is the FN+ cell stromal? The alternative view that the FN+ cells derive from mesenchymal or endothelial elements is supported by the absence of staining for GFAP, the lack of forms phenotypically intermediate between the FN+ cells and obvious glial cells, and the observation that in explant cultures these cells arise only as a late

phenomenon.

Although some cell lines obtained from human gliomas do produce FN many do not (Sherbet et al, 1982; Lubitz et al, 1980) and experiments on rat glial precursors have failed to induce interstitial FN matrix formation (Lim et al, 1982) despite their producing soluble FN. Critical studies of the FN-producing cells derived from normal human brain, and identified as glial (Ponten et al, 1969; Vaheri et al, 1976), have concluded that they are actually meningeal (Rutka et al, 1986a). Similar studies of fetal mouse cerebellum have concluded that such cells are endothelial, fibroblastic or meningeal in nature (Ghandour et al, 1982). Studies on the effect of differentiating agents on cell lines that did not express GFAP failed to induce GFAP expression and the authors conceded the possibility that this failure was due to the fact that the cells may have been of endothelial origin (Geder et al, 1987).

If the FN+ cells do not arise from neoplastic glial elements explanations are required for their quasi-transformed properties: their abnormal in-vitro growth patterns, their chromosomal abnormalities (Davenport et al, 1987; Kennedy et al, 1987), and the development of continuous cell lines some of which are tumourigenic (Jacobsen et al, 1987, Rutka et al, 1987a).

Although vascular and mesenchymal tissue in the brain does

not usually participate in the repair process following ischaemic damage to the same extent as is seen in non-neural tissue, after appropriate stimulation it will do so. Thus fibroblast and vascular proliferation and the formation of typical granulation tissue is characteristic of abscesses, and occasionally of deep haematomas (Masuzawa et al, 1985). The vascular proliferation that is seen in malignant astroglial tumours is sufficiently characteristic to be of diagnostic value (Rubinstein, 1972) but the fact that it may also be seen in relation to metastatic tumours (Feigin et al, 1958) indicates that it is an intrinsic capacity of cerebral vessels rather than a glioma-specific phenomenon. The vessels which form are often abnormal in the degree of maturation which they show, and as a consequence are often thin walled and liable to haemorrhage. Whether these abnormalities of structure result from the rapidity of growth or reflect acquired cellular defects resulting in defective vascular maturation is not known, but there is evidence that suggests that non-parenchymal elements (mesenchymal and vascular) in tumours may not be normal.

In human gliomas, although proliferating endothelial cells have been found to be diploid (Davenport et al, 1987a) the tumour cells in four out of 11 high grade gliomas studied were also diploid emphasising that diploidy does not necessarily equate with functional or genotypic normality. In an experimental virus-induced glial tumour a high

labelling index was observed in the endothelium and the possibility of direct transformation by the causative virus was discussed (Groothuis et al, 1980). In an autoradiographic study of glioma explants the majority of labelling was thought to be within the non-glial components (Haynes et al, 1978) and more recent work has shown that explants in which FN+ cells predominate are likely to be aneuploid (Davenport et al, 1987). To this evidence from glial tumours can be added direct observations of behavioural abnormalities in vitro of stromal components from other tumours.

Stromal fibroblasts from breast tumours have been found to show abnormal patterns of growth in-vitro which closely resemble the "heaping-up" of FN+ cells seen in glioma cultures (Delinassios, 1983); both tumour stromal fibroblasts and skin fibroblasts from the same patients have been found to show abnormal migration patterns in-vitro that are similar to transformed cells (Durning et al, 1984), and similar phenotypic abnormalities have been described in fibroblasts from patients with familial cancers (Kopelovich et al, 1979) or their relatives (Haggie et al, 1987). Although these findings do not mean that tumour stromal cells are transformed they do mean that phenotypic properties typically seen in transformed cells may be observed in non-parenchymal tumour-derived cells. It is possible that these properties may reflect abnormalities of gene expression, either hereditary or

acquired (conceivably as a result of the action of the carcinogen responsible for the transformation of the parenchymal cells), which may render the cell more liable to further alteration, and even eventual full transformation, if appropriate conditions apply either in-vitro or in-vivo. Thus the development from gliomas of tumourigenic cell lines with mesenchymal properties such as interstitial collagen deposition (Rutka et al, 1987a) or sarcomere formation (Jacobsen et al, 1987) could be seen as the result of further mutations occurring in vitro in an already susceptible cell type. Examples of such in-vivo progression are encountered in the central nervous system in the form of the gliosarcoma, a tumour in which neoplastic astroglial elements are combined with neoplastic mesenchymal elements, which may show striated muscle differentiation (Barnard et al, 1986), in a "mixed tumour" (Rubinstein, 1956; Russell et al, 1977). Sarcomatous elements have been estimated to occur in from 2.3% to 5% of all glioblastomas (Feigin et al, 1958; Morantz et al, 1976) which would be far higher than that encountered in most carcinomas (Willis, 1948). This would be in keeping with the hypothesis that the exuberant vascular proliferation so characteristic of malignant glial tumours is associated with an increased risk of transformation and subsequent tumour development in the mesenchyme (Feigin et al, 1958; Pena et al, 1973); indirect support for the importance of cerebral vessel responsiveness as opposed to glial tumour stimulation

comes from cases of sarcomas developing in relation to non-glial tumours metastatic to the brain (Feigin et al, 1984; Schwarz et al, 1963). Immunohistochemical studies on gliosarcomas have suggested that the sarcomatous element may be more closely related to the endothelial component of the proliferating vessel than the associated mesenchyme (Slowik et al, 1985) although others have argued that the sarcoma arises from non-endothelial cells (McComb et al, 1982).

The finding of large numbers of macrophages in relation to the vessels and sarcomatous areas of human gliosarcomas (Kochi et al, 1987) led to the suggestion that FN secreted by the macrophages was stimulating or guiding proliferating mesenchymal cells. This is of interest in the light of the suggestion that immune cells may provide the stimulus for the progression from hyperplasia to malignancy that characterises the vascular lesions of Kaposi's sarcoma (Brooks, 1986). Although in earlier studies on gliosarcomas in vitro no observations were made of antigenic phenotype (Slowik et al, 1985), later work has demonstrated that a cell line from such a tumour expressed FN, laminin and collagen IV and thus appeared to be genuinely mesenchymal in nature (Rutka et al, 1986). Mesenchymal properties have however been observed in cells cultured from glioblastomas without sarcoma (Rutka et al, 1987a; Jacobsen et al, 1987) and serial transplantation of vascular elements from malignant human gliomas has been

found to result in the development of sarcomas (Greene et al, 1968). This suggests that sarcoma development could simply be a progression from a state that may well exist exist in the vessels of many malignant gliomas. Against this view is the fact that GFAP+ and FN+ cells from such a tumour have been found to have similar chromosomal abnormalities (Westphal et al, 1988) which were held to indicate a common origin and would suggest that sarcomatous change is the result of phenotypic change in a malignant glial population rather than malignant change in stromal cells.

If the FN+ cells are derived from endothelium they fail to show the formation of primitive channels described in studies of endothelium from bovine capillaries, normal human foreskin and an ovarian tumour (Folkman et al, 1980). Differences in culture methods could however affect such findings since subsequent work demonstrated that channel formation required several weeks in primary culture (Folkman, 1984); as already described these conditions were found not to favour the appearance of FN+ FA cells from glioma explants.

The failure of the FN+ cells to express antigens normally associated with endothelial cells (Little et al, 1986) would appear to preclude their origin from endothelium; studies on endothelial cells however have shown that endothelial-associated antigens may be lost in-vitro

(DeBault, 1982) and this is in keeping with the findings from the present studies: cells bearing endothelial markers were not found after 14 days, and regular sheets of cells, whose appearances would suggest an endothelial nature, did not express endothelial markers. There is evidence from histological studies that the proliferating cells that comprise the hyperplastic blood vessels in malignant gliomas do not uniformly express endothelial markers (Moore et al, 1986a) although the authors of the study interpreted their findings as indicating the involvement of pericytes in the process.

Non-neoplastic endothelial cells have been found to undergo morphological and behavioural changes in-vitro which may explain some of these inconsistencies. A spontaneously arising variant cell derived from normal bovine endothelium shows more FN production than a normal endothelial cell and, instead of forming regular monolayers, grows over or under sheets of normal cells (McAuslan et al, 1980) in a pattern very reminiscent of the FN+ cells derived from human gliomas. Similar behaviour, including the development of anchorage-independent growth and enhanced plasminogen-activator activity during active growth, has been shown not to be associated with chromosomal abnormality (Laug et al, 1980) supporting the view that these are properties that can, in appropriate circumstances, be demonstrated by non-neoplastic cells.

Endothelial cells are not, however, the only cells related to vascular structures and involved in new vessel formation. In the context of vascular proliferation cells derived from smooth muscle cells of the vascular media have been found to function both as fibroblasts and as precursor cells of fibroblasts and smooth muscle (Wissler, 1967). Furthermore such cells appear to have survival advantages over fibroblasts in vitro, showing a longer period of logarithmic growth and reaching higher cell densities before their growth rate levels off (Burke et al, 1977). Detailed in vitro studies of smooth muscle cells, of vascular and non-vascular origins, have shown that they form aggregates 2-4 days after growing from an explant (Kasai et al, 1964) and after approximately one week can be seen to pile one upon the other growing in multiple overlapping layers (Ross, 1971). These patterns of behaviour are very similar to those observed among FA cells derived from the tumour cultures in the present studies. FA cells could therefore be cells of vascular origin with properties that prevent precise definition as endothelial, fibroblastic or smooth muscle. The stimulus to their growth and behaviour could derive from the neoplastic glial cells in vivo, and in vitro conditions could provide an environment that allowed expression of their unexpected combination of properties. PDGF, which is produced by some glioma cells in vitro, has been mooted as one mediator of tumour-induced vascular proliferation (Westermark et al, 1985) but the only response of

endothelial cells to PDGF is an increase in prostacyclin synthesis not proliferation (Coughlin et al, 1980). Intriguingly smooth muscle cells and fibroblasts possess PDGF receptors and do respond by proliferation, a property also demonstrated by some glioma-derived cell lines (Heldin et al, 1981). The possibility of an interplay between components of the vascular wall has been extended to suggest that transition may occur between endothelial cells and fibroblasts in reactive (Beranek et al, 1986) and neoplastic processes (Slowik et al, 1985). An ultrastructural study of a gliosarcoma (Kishikawa et al, 1986) has suggested that the cells comprising the sarcomatous component are myofibroblastic or pericytic in nature.

Medial smooth muscle cells, their precursors or the postulated multipotent subendothelial vasoformative reserve cell (Stein et al, 1969) may therefore be in a state in tumour vessels that would allow them to preferentially grow and survive in culture. If the vessel were to be disrupted by collagenase prior to culture such growth might be enhanced when compared to the situation in a vascular fragment in an intact, largely glial, explant. Such an explanation would fit with the observation that FA cell predominance is more likely to occur in dispersed cell cultures than in explants.

Conclusions from in vitro studies

It is not easy at this stage to form a unitary hypothesis concerning the origin of the FN+ cells which constitute one component of the heterogeneous populations observed in these in-vitro studies on human gliomas. It is apparent that culture method is a more important determinant of their development than tumour grade. They may be transformed cells, and there is certainly evidence that fully transformed cell lines can develop from them, but they could in many cases be cells which exhibit behaviour interpreted as transformed but which is in fact normal for endothelial, or at least vascular precursor, cells. The possibility that they are showing phenotypic characteristics in-vitro that are not apparent in-vivo does not preclude a glial derivation but the evidence that neoplastic glial cells can produce FN is often indirect and open to alternative explanation.

In a search for cells showing the antigenic expressions of the cell types identified in-vitro attention was turned to the phenotypic characteristics of parenchymal and non-parenchymal cells in astroglial tumours.

CHAPTER 3. VASCULAR AND PARENCHYMAL ELEMENTS IN HUMAN GLIOMAS AS POSSIBLE SOURCES OF FIBRONECTIN-PRODUCING CELLS IN-VITRO.

INTRODUCTION

From the data presented so far it is apparent that one major cell type derived from gliomas in-vitro, namely the GFAP+ cell, both process-forming and non process-forming, is of glial origin. The second major cell type, the FN+ cell, has features which could be interpreted as transformed but its antigenic expression does not include the intermediate filament GFAP which would identify it as also being of glial origin. Nevertheless, as has been discussed, the lack of GFAP does not preclude a glial origin. The alternative hypothesis that this cell type could be of vascular origin has been considered, and receives support from changes known to occur in cells of vascular origin in response to appropriate stimulation (Laug et al, 1980; McAuslan et al, 1980). The latter hypothesis could be further explored by an examination of the phenotypic characteristics of vessel-associated and parenchymal elements in tissue sections from human tumours to determine the location and distribution of cells with antigenic profiles similar to those observed in-vitro.

Angiogenesis

The acquisition of a blood supply marks an important stage in the transition of a neoplastic proliferation from a group of cells, dependent on diffusion of nutrients and oxygen for survival and growth, to a system whose further growth can be sustained so long as new vessels are formed (Folkman et al, 1971; Gimbrone et al, 1972). Following this contact with vascular channels neoplastic cells possessing appropriate properties, such as invasion and motility, will be able to gain access to the circulation and thus metastasise to distant sites; nevertheless the establishment of distant tumours will depend on the ability of such cells to grow in the initially low cell densities that may result from such a process.
Angiogenesis consists of the formation of new vessels from existing blood vessels and is not a process peculiar to tumours. Indeed, analyses of tumour vasculature have suggested that the greatest vascular density is found at the edge of growing tumours indicating that the host vessels, rather than intratumoural vessels, are most responsive to tumour-derived angiogenic factors (Thompson et al, 1987), even though the concentration of such factors is probably higher in the tumour centre than at its edge. Whether this reflects an abnormality of responsiveness of the constituent cells of the tumour vessels, or an absence from their structure of components necessary for an organised response, is unclear. The latter possibility would accord with the observed

structural abnormalities observed in many vessels in malignant gliomas that are believed to be the source of the haemorrhages frequently seen in such lesions (Liwnicz et al, 1987).

Angiogenesis is a feature of normal growth and repair where it constitutes an integral part of a controlled process that is normally self-limiting. As an example the rich vascularity that characterises granulation tissue formed during the repair of a wound or an ulcer base diminishes as the repair process proceeds. Even in the angiogenesis that accompanies neoplastic growth there is a clear indication from labelling studies that many cells in the newly formed vessels are not proliferating, and this has been adduced as indicating a programmed cessation of growth akin to a maturation process (Nagashima et al, 1987). The production of new vessels by existing blood vessels can be initiated by a wide range of soluble factors but the process whereby the new vessels are formed does not appear to differ significantly with different stimuli.

The primary response of vessels to an appropriate stimulus is the proliferation and mobilisation of resting endothelial cells in capillaries or venules (Tannock, 1970; Warren et al, 1972). Not all endothelial cells are equally responsive and differences have been noted between cells from large and small vessels (Folkman et al, 1980;

Gross et al, 1983a; Hockel et al, 1987; Nagashima et al, 1987; Zetter, 1980), and some sprouting can occur as a result of cell migration alone without active proliferation (Sholley et al, 1984). Activation of endothelial cells is associated with protease production (Gross et al, 1983a), disruption of their basement membranes and migration towards the source of the stimulus (Zetter, 1980).

Endothelial cells have been found to produce plasminogen activator (PA) (Loskutoff et al, 1977) in-vitro and the morphological features of endothelial cells associated with such production (McAuslan et al, 1980) have already been referred to. The plasmin produced by the activity of such PAs has broad proteolytic properties, degrading both fibronectin (FN) and laminin, and can also result in collagen degradation by activation of latent local collagenases (Schor et al, 1983). The finding of large amounts of FN in relation to proliferating capillaries (Clark et al, 1982), the observation that FN may direct cell migration in-vitro (as discussed in Chapter 2, pg93) and studies of its enhancing effects on endothelial cells in-vitro suggest that it may provide a substrate in-vivo on which endothelial cells may be better able to migrate and proliferate (Gospodarowicz et al, 1980).

The migration of endothelial cells appears to take place as cohesive cords of cells rather than as single separated cells (Schoefl, 1963; Schor et al, 1983) and it is the subsequent canalisation of these cords that gives rise to

new vessels. It is apparent that the stimulating factors do not operate only on the endothelial cells since other mesenchymal components (pericytes and fibroblasts) also proliferate (Molne et al, 1987; Tannock, 1970; Warren et al, 1972).

The newly formed vessels undergo a process of maturation: basement membrane is laid down and an adventitial component is acquired consisting of fibroblasts and pericytes (possibly derived from medial fibroblasts or undifferentiated precursors). These also appear to contribute to the formation of the basement membrane. Control of this process of recruitment and maturation is complex and ill-understood, but there is in-vitro evidence that endothelial cells can produce factors that are chemotactic for smooth muscle cells (Harris-Hooker et al, 1983), and that Platelet Derived Growth Factor (PDGF) produced by endothelial cells may induce proliferation of fibroblasts and smooth muscle cells, both of which bear PDGF receptors (Barrett et al, 1984, Rubin et al, 1988).

Cellular behaviour is known to be affected by a cell's relation to its substrate and it has been postulated that this is achieved by alterations in membrane expression of growth factor receptors (Gospodarowicz et al, 1978). The relationship between a cell and its substrate may be radically altered when it develops the capacity to synthesise basement membrane; this may be an important stage in both achieving structural integrity for the newly

formed vessel and in terminating the proliferative process. Although the process of maturation described above is commonly observed in vivo, capillary endothelial cells do have the capacity to form new vessels in vitro without the involvement of other cells (Folkman et al, 1980).

Although new vessels may arise predominantly from intrinsic vascular elements there is evidence to indicate that the final structure of tumour-associated vessels is determined in part by the nature of the stimulus. Defective development of medial and adventitial layers in vessels in the centre of experimental tumours (as opposed to those at the periphery) has been attributed to abnormal blood flow through them (Warren et al, 1966), but in other studies the pattern that resulted was found to be peculiar to a given tumour type rather than vessel position (Goodal et al, 1965). The latter observations, and the finding of endothelial features similar to those of the kidney in a metastatic renal carcinoma (Hirano et al, 1972) indicate that, in the process of neovascularisation, growing parenchymal cells can effect considerable control over other cell populations. Nevertheless abnormalities of maturation might also be attributable, in part, to acquired defects in the angiogenic cells from which the new vessels derived.

Numerous stimuli to angiogenesis have been described. Implants of intact tissue, both malignant (Goodal et al, 1965; Tannock, 1970; Warren et al, 1966; Warren et al, 1972) and normal (Molne et al, 1987) elicit a vascular response from appropriate hosts. In some experimental systems there appears to be a direct relationship between the capacity of a particular tissue to induce an angiogenic response and the extent to which the component cells have been altered by a carcinogenic agent (Maiorana et al, 1978). A wide range of naturally or virally transformed cells produce angiogenic factors in vitro (Klagsbrun et al, 1976; McAuslan et al, 1979) and, significantly, such cells may still show residual features of normality (such as density-induced growth inhibition). These findings emphasise the point that angiogenic capacity is not restricted to fully transformed or neoplastic cells. Endothelial growth factors have been derived from cultures of human gliomas (Hirschberg et al, 1984; Libermann et al, 1987) and from tissue extracts of a number of human CNS tumours including a single glioblastoma (Lye et al, 1986). Oligosaccharides derived from the breakdown of hyaluronic acid have been proposed as the angiogenic stimulus in diabetic retinopathy (West et al, 1988) and a similar mechanism may underly the observed angiogenic capacity of synovial fluid from non-inflammatory arthropathies (Brown et al, 1983). Macrophages, both those activated in-vitro (Koch et al, 1986; Leibovich et al, 1987; Ooi et al, 1983; Polverini et

al, 1987) and those associated with an experimental sarcoma (Polverini et al, 1984), lymphocytes, either directly or by activating macrophages (Auerbach et al, 1979), and neutrophils (Fromer et al, 1975) have all been shown to produce angiogenic substances. The possibility that endothelial cells play a central role in the induction and control of the process is strengthened by the observation that they can utilise serum components to produce factors that enhance their own growth and that of other mesenchymal cells in vitro (Gajdusek et al, 1982), and can induce new vessel formation (Harris-Hooker et al, 1983) in vivo.

Studies of the active components isolated from a variety of sources have revealed a range of soluble angiogenic factors with varying properties. In an analysis of vasogenic agents BenEzra made the useful conceptual distinction between those that induce the migration of endothelial cells such as prostaglandins and copper, and those that induce mitogenesis. He also expressed the reservation that the demonstration of activity in vitro was not necessarily associated with equivalent in vivo activity (BenEzra, 1978).

Tumour Angiogenesis Factor (TAF) was first identified (Folkman et al, 1971), in a crude tumour extract. Further purifications yielded a low molecular weight (210kD) Endothelium Stimulating Factor (ESF) (McAuslan et al,

1979) which appears to mobilise endothelium by virtue of the copper it contains but does not actually stimulate proliferation. Copper, especially in association with heparin, is a potent stimulator of endothelial cell migration (Gullino, 1986) and the effect of mast cell-derived heparin (Azizhan et al, 1980) can be inhibited by protamine (Taylor et al, 1982). The action of copper in vitro can be further enhanced by the presence of FN which seems to act not as a chemo-attractant but rather by enhancing cell attachment to the substrate via cell surface gangliosides. The cumulative effect of a combination of heparin and FN can be further increased by using cleavage fragments of both, of the sort likely to be produced locally by the action of proteases of endothelial origin (Gullino, 1986). Evidence that similar interactions may be operating in vivo is found in the correlation that is observed between FN levels and endothelial cell proliferation in healing skin wounds in guinea-pigs (Clark et al, 1982).

Endothelial proliferation has been related to the action of a number of growth factors. These include Endothelial cell Stimulating Angiogenic Factor (ESAF) (Schor et al, 1983), Fibroblast Growth Factor (FGF) (Gospodarowicz et al, 1986; Hayek et al, 1987), Epithelial Growth Factor (EGF) (Schreiber et al, 1986) and Transforming Growth Factor (TGF) (Schreiber et al, 1986,Folkman et al, 1987). ESAF, which does not contain copper and requires the

presence of type I collagen to be effective in vitro (Schor et al, 1980), has been found with a molecular weight of 600 in tissue extracts from meningiomas, neurilemomas and a glioblastoma (Lye et al, 1986). The requirement for the type of collagen that is typically associated with interstitial tissues may reflect its role as a binding site for ESAF, or as a modulating factor determining, in some way, the expression on the cell surface of receptors for specific stimulating factors (Gospodarowicz et al, 1978). During this phase of proliferation endothelial cell migration will be through tissue containing type I collagen in contrast to the resting state when the predominant contact will be with the type IV collagen of basement membrane. It is therefore of interest that the production of basement membrane by maturing endothelial cells in new vessels is enhanced by contact with type I collagen (Schor et al, 1983). The maturation of vessels could therefore be seen to be, in part, dependent on the concurrent proliferation of fibroblasts and other cells capable of synthesising type I collagen and might arguably be delayed if new vessel formation is taking place into tissues, such as the brain, which are poor in interstitial collagen. The interplay between these different factors is obviously complex; it seems likely that some factors that are capable in their own right of affecting endothelial cells in vitro may exert their effect in vivo by stimulating the release of a final active factor from another cell type. Thus TGF and

FGF may have more influence by their effect on fibroblast production of extracellular matrix (Gospodarowicz et al, 1986; Schreiber et al, 1986) or by stimulating macrophages to release angiogenic factors (Folkman et al, 1987). Further examples of such interactions come from studies of Tumour Necrosis Factor (TNF) which is secreted by activated macrophages. In vitro TNF is cytostatic for endothelial and smooth muscle cells and acts as a non-competitive antagonist of FGF; in vivo it acts as a vasogenic stimulus by virtue of its capacity to induce a local inflammatory response (Frater-Schroder et al, 1987).

The role of the extracellular matrix and non-endothelial cell elements in control of the angiogenic process has already been mentioned; recent observations have however suggested that interferons, which are more typically associated with the cellular interactions and control of proliferation that take place in the cellular immune response, may also have a controlling function in angiogenesis (Sidky et al, 1987). The angiogenic response to implanted tumour, or to injected lymphocytes, was inhibited by interferon but the effect was species specific and was less marked for tumour-induced angiogenesis. This observation suggested that interferon was operating by an action on angiogenic factor release which was more effective on lymphocytes, rather than by directly inhibiting mobility or proliferation of endothelial cells.

Plasminogen Activator (PA)

The association of PA secretion with neoplastic transformation has been discussed in Chapter 1. This association has been thought to relate to invasive capabilities conferred on the cell by such secretion (Pauli et al, 1987). However doubts have been cast on the significance of PA and its value as a marker of malignancy by the finding of an inconsistent relationship with the induced transformed state (Wigler et al, 1976) and by studies of the mode of action of inducing agents (Plagemann et al, 1980; Rovera et al, 1977). The latter studies showed that these acted on basic cell processes such as differentiation and hexose transport while the induction of PA appeared to vary as much with cell type as with development of a transformed state and was probably epiphenomenal. PA production was shown to be a property of endothelial cells (Levin et al, 1982; Loskutoff et al, 1977; Soreq et al, 1981; Takashima et al, 1969; Todd, 1959) and could be induced in such cells by the same tumour promoters shown to act on other cell types. Furthermore studies on endothelial cells showed that the matrix degradation and collagenolysis on which the invasive properties of PA-producing cells were thought to depend were not dependent on PA production (Laug et al, 1985).

Two distinct groups of plasminogen activators are recognised: urokinase (UK), so named because of its

presence in urine, and tissue associated (t-PA), which has been isolated from a variety of normal and abnormal tissues.

UK appears to have two components, with molecular weights of 31.5kD and 54.7kD (White et al, 1966). The latter was originally thought to be a complex of the lower molecular weight component with an inert carrier protein (White et al, 1966) but it is now accepted as a distinct heavier chain (Soberano et al, 1976).

Although estimates of the molecular weight of t-PA vary between 72kD for human melanoma (Rijken et al, 1981) and 68kD for human uterine t-PA (Rijken et al, 1979) other estimates have yielded values in the range 52.5 (for pig heart) to 80kD (Reddy et al, 1980a) and 67kD (Binder et al, 1979) (for human cadaveric vascular trees). There is however general agreement that the molecule is separable into two subunits with molecular weights variously estimated at 33 and 39kD (Rijken et al, 1981) or 31 and 38kD (Rijken et al, 1979).

Many tumour cells have been found to produce UK in vitro (Reddy et al, 1980a; Rijken et al, 1981) but studies on animal and human glial tissues and tumours have yielded rather confusing results. Normal mouse astrocytes were found to secrete t-PA (Toshniwal et al, 1987, Toshniwal et al, 1987a) although in-situ fibrinolytic activity in rodent and human brain was largely found in endothelium, choroid plexus and meninges (Soreq et al, 1981; Takashima

et al, 1969). Short term cultures of both normal and neoplastic glial tissue yielded fibrinolytic activity (Wilson et al, 1978) but the cells responsible were not characterised. Subsequent analyses showed that three out of four glioblastomas studied produced UK and one produced a PA that was distinct from UK although again no attempt was made to characterise the cells responsible for this activity (Wilson et al, 1980). Cloned cells from a human glioblastoma, which were S-100 positive and thus thought to be of neural origin, also produced PA that was distinct from UK (Tucker et al, 1978). More recently PA activity of human glioma cell lines has been found to be UK (Dano et al, 1982) and to correlate with tumourigenicity (Gross et al, 1988) and studies on short term cultures found PA activity, not characterised as UK or t-PA, to correlate inversely with the expression of glial characteristics (Frame et al, 1984; McLean et al, 1986). In the latter studies PA was used as a direct marker of malignancy and in an extension of this PA from wet tissue has been assayed without characterisation and in the belief that it is derived from neoplastic cells (Quindlen et al, 1987). The problem is compounded by the finding that discrepancies may occur between the type of activator found in solid tumour tissue and that synthesised by cells cultured from the same tumour (Markus et al, 1984).

There appear, therefore, to be several uncertainties:
i) can PA activity alone can be used as an in-vitro marker of malignant glial cells (Frame et al, 1984; McLean et al, 1986)?
ii) what is the nature of the cells responsible for PA activity, rather than UK, in cultures from some human gliomas (Tucker et al, 1978; Wilson et al, 1980)?
iii) what possible sources could there be for this activity in the original tumours (Quindlen et al, 1987)?

The importance of proteolytic activity in the early stages of angiogenesis has been mentioned; tissue fibrinolytic activity was localised to vascular endothelium nearly 30 years ago (Todd, 1959), and subsequently identified in the endothelium of normal brain vessels (Takashima et al, 1969, Soreq et al, 1981). Few histological studies have been carried out of the distribution of PA in human tumours but Kohga and colleagues (Kohga et al, 1985) compared t-PA with UK in colonic cancer and found the former in stroma and vessel endothelium and the latter in tumour cells. The availability of a monoclonal antibody to t-PA provided an opportunity to combine an examination of other characteristics of vessel-associated elements in gliomas with a study of the localisation of t-PA in such tumours. Such a study was seen as a means of helping to determine whether t-PA-producing cells in cultures from human gliomas could have originated from tumour parenchyma or from elements of the fibrovascular stroma. A series of

human central nervous system tumours was, therefore, studied with particular reference to those elements in malignant gliomas of non-parenchymal origin: proliferating blood vessels and mesenchymal cells associated with them.

MATERIALS AND METHODS

The tissue examined in this study derived from diagnostic specimens received in the Neuropathology Laboratory, University of Leeds between 1983 and 1987. Fresh tissue was frozen in isopentane or directly in liquid nitrogen and examined after storage periods ranging from 36 hours to 3 years.

When this section of the work was carried out material was available from four low grade astrocytomas (including one gemistocytic astrocytoma), 22 malignant astrocytomas, (20 grade IV and 2 grade III), three meningiomas, three metastases, two pieces of cortex (one normal and one from vicinity of a malignant astrocytoma), two choroid plexus papillomas, one ependymoma, one ganglioglioma, one oligodendroglioma and one astroblastoma.

Cryostat sections were cut at 8µm, mounted on poly-L-lysine coated glass slides, air dried overnight and fixed for 15 min in Acetone. After pre-treatment with normal goat serum (NGS) diluted 1:5 in Tris buffered saline (TBS) to block non-specific binding sites sections were stained for 1h with the monoclonal antibody ESP6 (Bioscot, Edinburgh), which recognises cell-associated t-PA but not urokinase, diluted 1:2 in NGS (diluted 1:20 in TBS) followed by a goat anti-mouse fluorescein conjugate diluted 1:50 in NGS (diluted 1:20 in TBS). Double staining was then carried out with a rabbit

anti-human polyclonal antibody applied for 30 min, followed by a swine anti-rabbit rhodamine conjugate (diluted 1:50 in TBS) for 30 min.

One or more of the following polyclonal antibodies were used for each tumour:
Glial Fibrillary Acidic Protein (GFAP) (Dakopatts; diluted 1:200 in TBS)
Factor 8 Related Antigen (F8RAg) (Dakopatts; diluted 1:100 in TBS)
Fibronectin (FN) (Dakopatts; diluted 1:200 in TBS)

Other sections were double stained with Ulex europaeus 1 lectin (UEl) conjugated with FITC (diluted 1:50 in phosphate buffer) although for these the t-PA was demonstrated with a rhodamine rather than a fluorescein conjugate.

Sections from all tumours were stained with ESP6 and F8RAg, and ESP6 and UE; sections from the astrocytic tumours were also stained with ESP6 and Fibronectin, ESP6 and GFAP, vimentin and desmin, in the case of the latter two antigens using the same monoclonal antibodies detailed in Chapter 2, double stained with the polyclonal anti-GFAP. The final preparations were viewed under fluorescent light using mean exciting wavelengths of 490 nm and 540 nm for fluorescein and rhodamine respectively. Sections of full-term placental bed were used as positive

controls for t-PA, EU1 and F8RAg, a glioblastoma provided positive control for FN, GFAP and vimentin, and human myometrium was used as a control for desmin staining. Specificity of UEl staining was checked by co-incubation with a 0.1M solution of alpha-L(-) fucose which bound the lectin and abolished positive staining in the tumour and control sections.

Western Blot.

To assess the affinities of the ESP6 (Bioscot) antibody a western blot was performed on an extract of protein from a glioblastoma multiforme.

The protein extraction was carried out from one gram of glioblastoma multiforme tissue by grinding the tissue under liquid nitrogen and then adding lml TES buffer (20 mM Tris HCl ph 7.8, 90 mM NaCl, 1 mM disodium EDTA) containing 0.1% Triton X100. The resulting homogenate was covered with a thin layer of liquid paraffin and centrifuged at 40000 rpm for 1h at 4°C.

Protein separation was by discontinuous SDS gel electrophoresis (Laemmli, 1970) using 10% polyacrylamide gels with duplicate lanes of test material and molecular weight standards (12.3, 18.4, 25.7, 43, 68, 92, and 200kD). The separated proteins were visualised with Coomassie blue and the test material transferred by blotting to nitrocellulose sheet (Towbin et al, 1979).

Final visualisation was with a three layer immunoperoxidase method using repeated washes of the diluting buffer (Tris buffered saline - TBS) between stages:

i) Non-specific binding sites were blocked by pre-treatment with normal goat serum (NGS) diluted 1:5 for 1h at 37°C

ii) Mouse monoclonal antibody ESP6, diluted 1:5, for 2h at room temperature

iii) Rabbit-anti-Mouse IgG, diluted 1:50, for 45 min at room temperature

iv) Goat-anti-Rabbit Peroxidase (Biorad), diluted 1:500, for 45 min at room temperature.

v) Develop in 4-chloro-napthol.

From a plot of log Molecular Weight against distance migrated by the known standards and direct measurement of the distances migrated by bands stained on the blot the molecular weights of the stained proteins were calculated.

Tissue cultures

As an adjunct to the studies carried out on the tissue sections described above cell preparations from two high grade astrocytomas were also examined immuno-histochemically for t-PA activity. The methods used were identical to those described for the tissue sections with the exception that fixation was with ethanol/ether as described in Chapter 2. The cultures derived from material already described in the previous section and consisted of

explants from one case after 8 days in primary culture and two preparations from a different tumour 8 days after first passage and a total of 28 days in-vitro.

RESULTS

The nitrocellulose blot demonstrated four bands of reactivity (fig.3.1): a very faint one at 72kD, a defined band at 55kD, and two less defined bands centred on 41 and 31kD.

Control sections from placenta stained for t-PA showed variable staining of villous trophoblast, consistent staining of extra-villous trophoblast and patchy staining of the vascular endothelium (fig.3.2). In contrast only the endothelium showed staining for F8RAg or with UEl (the latter staining being abolished by prior incubation with fucose (fig.3.3)).

Malignant gliomas and normal or adjacent brain

Parenchymal cells

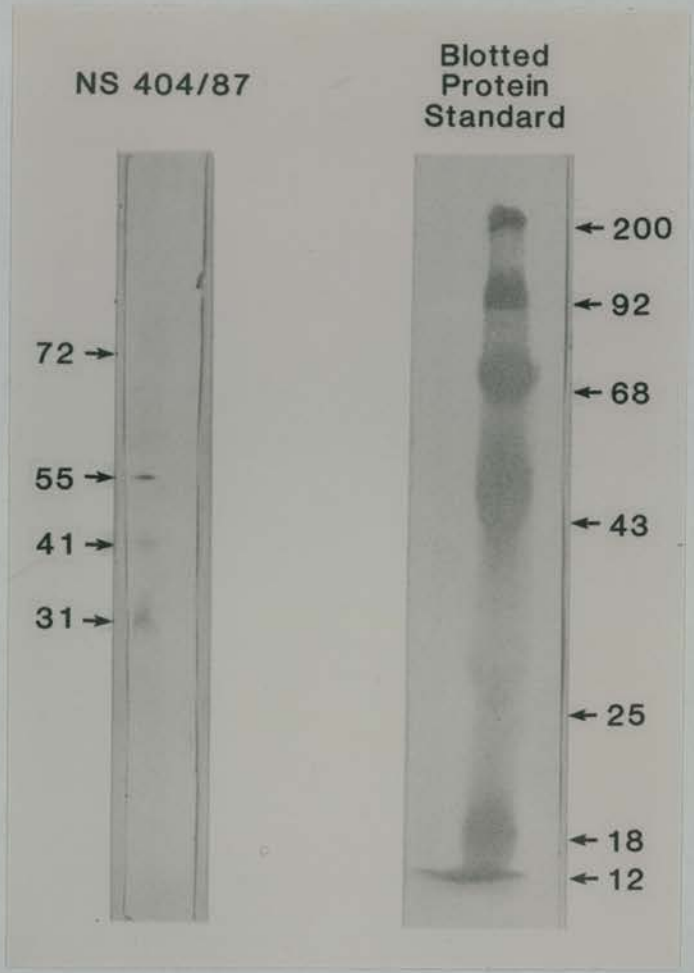
Process-bearing cells constituted the majority population in most of these tumours and in general the expression of intermediate filaments was far greater in the processes than in the cell bodies.

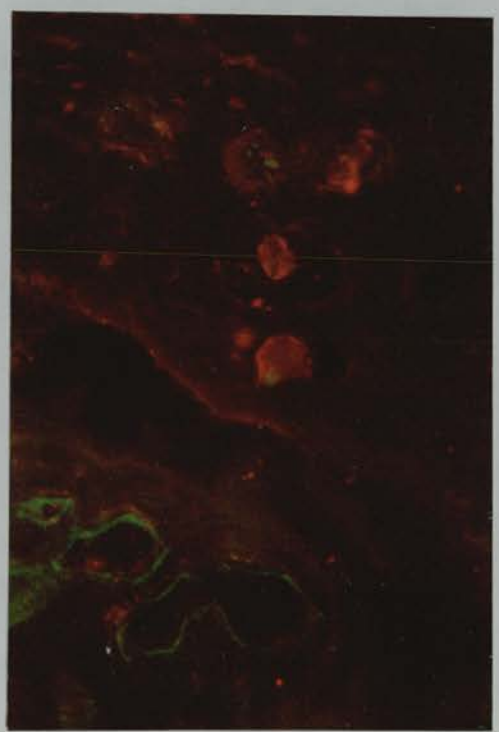
All the tumours examined expressed GFAP in the majority of the cell processes and similar expression of vimentin was also found (fig.3.4). Desmin was much less widespread but was present in some stellate cells in most tumours (fig.3.5). This was in marked contrast to the intense expression found in three desmoplastic lesions (see below).

Fig.3.1 Western blot of protein extract from glioblastoma multiforme (left) showing bands at 55kD, 41kD and 31kD. Right hand strip shows protein of standard molecular weights. See text for interpretation.

Fig.3.2 (left) Placental bed showing UEl staining of endothelium (green) and t-PA (orange) in extra-villous trophoblast and endothelium (beneath surface UEl reaction). Cryostat section, direct EUl, monoclonal anti-t-PA, x520

Fig.3.3 (right) Placental bed showing no reaction of endothelium with UEl after preincubation with fucose. Cryostat section, x260


NS 404/87
Blotted
Protein
Standard
72
55
41
31
200
92
68
43
25
18
12



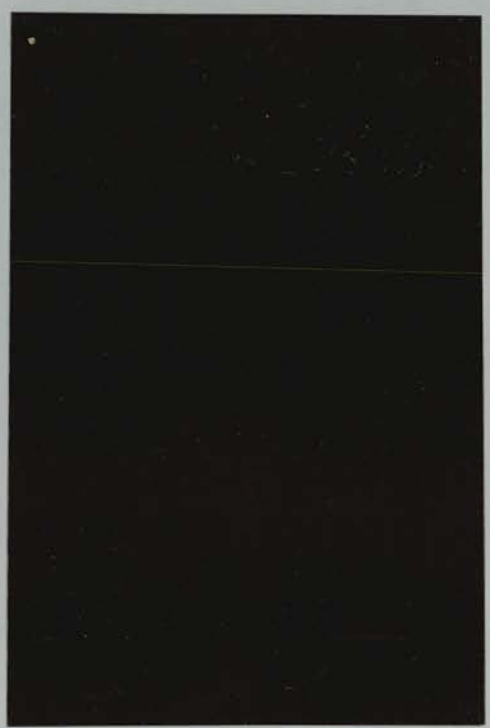
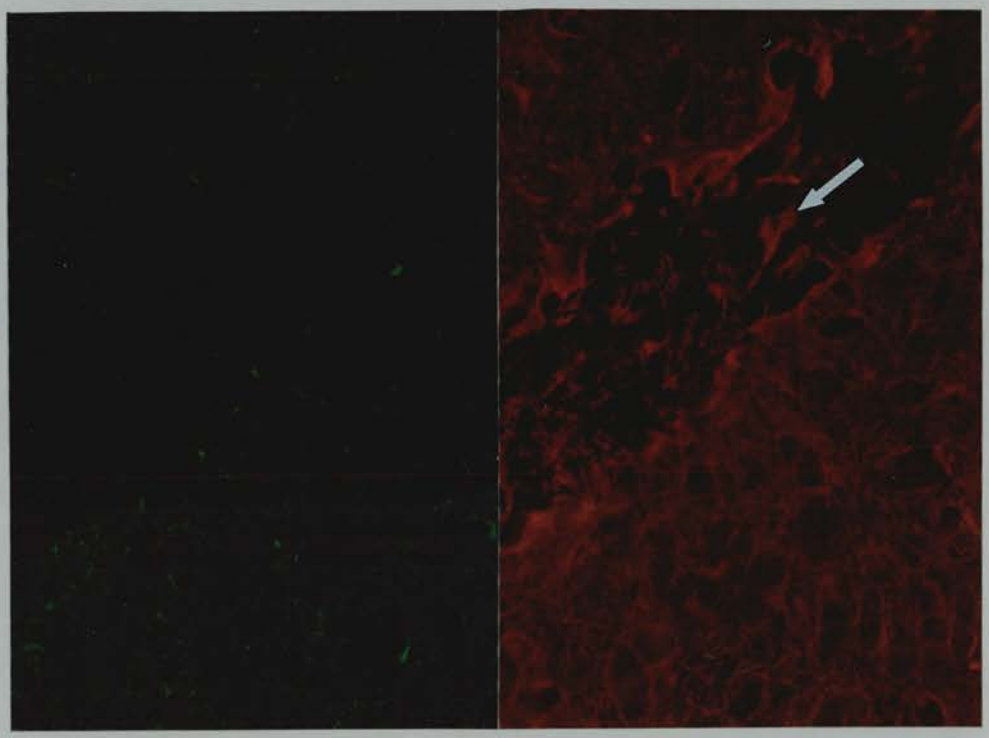
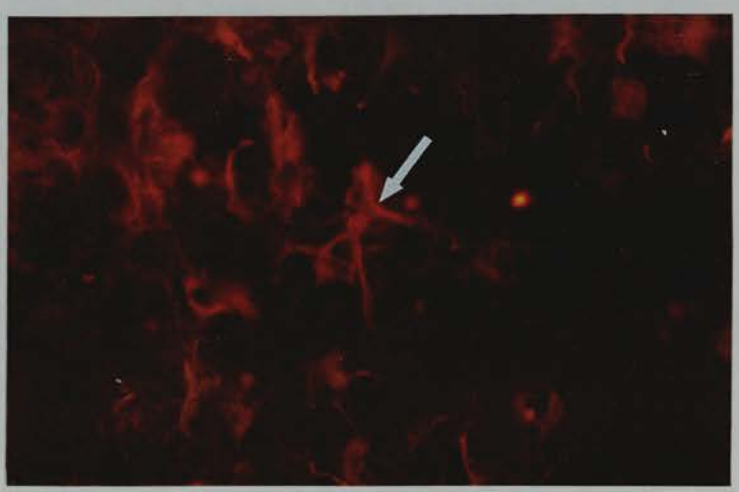


Fig.3.4 The distribution of vimentin (left) corresponds with that of GFAP (right) except where cell processes extend into the perivascular zone (arrow). High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal anti-vimentin, x520

Fig.3.5 Process-forming glial cells stain strongly for desmin (arrow). High grade astrocytoma, cryostat section, monoclonal anti-desmin, x520





Interposition of cell processes into FN+ areas associated with blood vessel walls was common but, while they usually stained for GFAP, staining for vimentin was much less consistent (fig.3.4); only very occasionally were single FN+ cells, that did not form small channels, seen outside the confines of vascular areas (fig.3.6). Such cells did not express GFAP or F8RAg.

Stellate astrocytes in normal and infiltrated cortex stained with GFAP, vimentin and sometimes with desmin, although cells whose size and arrangement suggested a neuronal nature also showed faint staining with desmin.

The presence of t-PA in tumour cells (expressing GFAP) was found in only one Grade IV astrocytoma (fig.3.7a) which was characterised by plentiful giant cells (fig.3.7b) although the reaction did not seem to be limited to them. In a few tumours occasional single round cells, lacking processes and not staining with GFAP, showed intense staining for t-PA (fig.3.8).

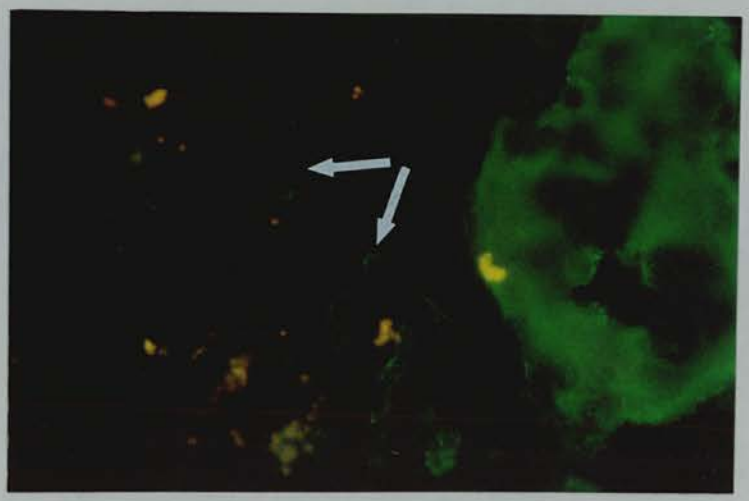
Endothelium

The cells lining blood vessels showed uniform positive reaction of the cell surface with UEl. Both the basal and luminal surfaces of the lining endothelium stained with FN (fig.3.9) although basal staining was often much stronger. Reaction for F8RAg was generally granular and cytoplasmic (fig.3.9) although in the larger vessels, typically those not associated with glomeruloid proliferations, which may in some cases have been pre-existing vessels in

Fig.3.6 Small FN+ profiles adjacent to a blood vessel (arrows) could be single cells, or, more likely, small vascular sprouts. High grade astrocytoma, cryostat section, monoclonal anti-FN, x520

Fig.3.7 (left) Glioblastoma multiforme showing focal t-PA reactivity which is predominantly on cell surfaces. Cryostat section, monoclonal anti-t-PA, x520

(right) Same tumour showing plentiful giant cells, although the t-PA reactivity corresponded more with the smaller cells. Cryostat section, H&E, x200



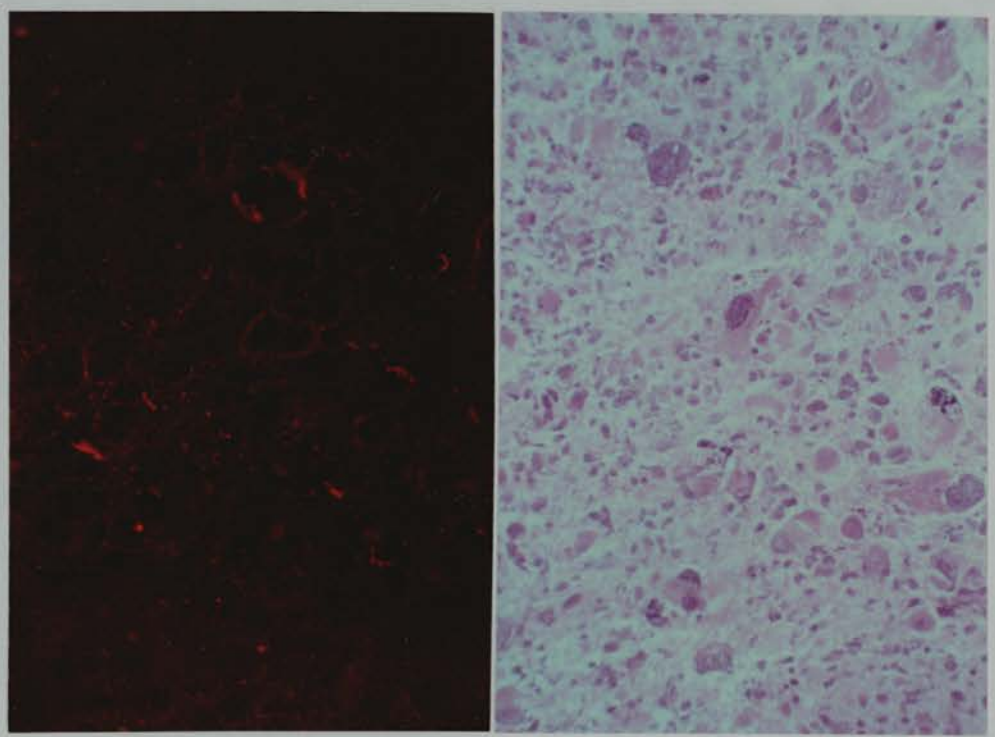
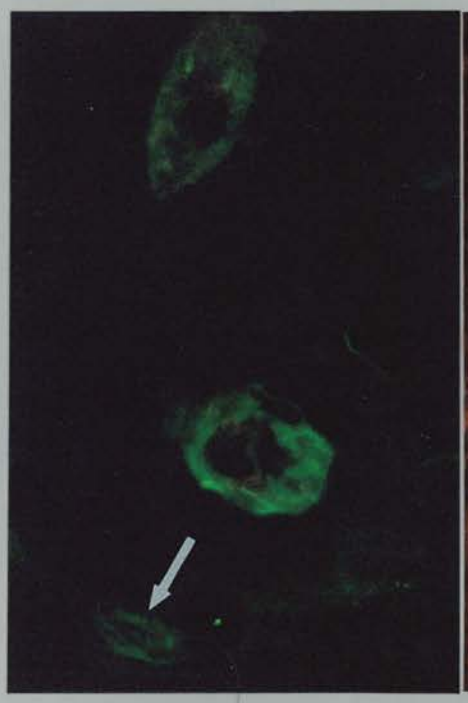
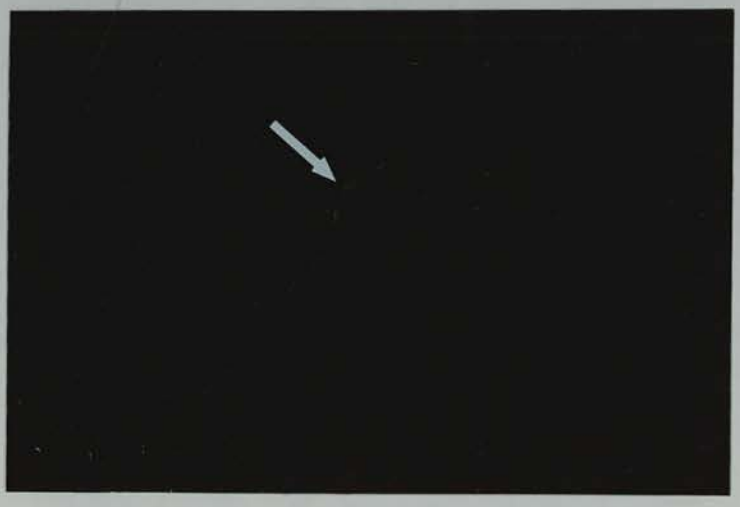


Fig.3.8 Single cell in tumour parenchyma staining strongly for t-PA. High grade astrocytoma, cryostat section, monoclonal anti-t-PA, x520

Fig.3.9 Small tumour vessels show closely corresponding staining for FN (left) and F8RAg (right). One small vessel (arrow) shows staining with FN but not F8RAg. High grade astrocytoma, cryostat section, polyclonal anti-F8Rag, monoclonal antiFN, x520

Fig.3.10 Vimentin is present in the thin endothelium (arrow) of a relatively large vessel. High grade astrocytoma, cryostat section, monoclonal anti-vimentin, x1040





infiltrated brain, expression was more restricted to the luminal surface. There was usually co-expression of F8RAg and FN in identifiable vascular structures (fig.3.9) although occasional small channels visible as slits with FN+ lining were F8RAg-. Cells in these areas were also positively stained with UEl.

In sections that were clearly derived from the interface between infiltrating tumour and brain there was a noticeable reduction in FN staining in the walls of residual normal cerebral vessels when compared to the new vessels of the tumour, and F8RAg was more consistently restricted to the luminal surface. Vimentin expression varied with vessel size and was generally more obvious, and sometimes restricted to, larger vessels with thinner endothelium (fig.3.10). As a consequence many small vessels composed (presumably) solely of endothelial cells were negative for vimentin. In all cases the vimentin content of the tumour was generally greater than that of the endothelium.

No desmin positivity was recorded in any cells identifiable by location or staining reaction as endothelial.

In all the tumours examined, reactivity for t-PA, when present, was clearly localised in vascular endothelium, which was identified by co-expression of F8RAg or FN, or UEl staining, although the intensity of staining varied from case to case. The staining was of a granular or

punctate appearance, distributed largely within the cell cytoplasm although some surface activity was also resolvable (fig.3.11).

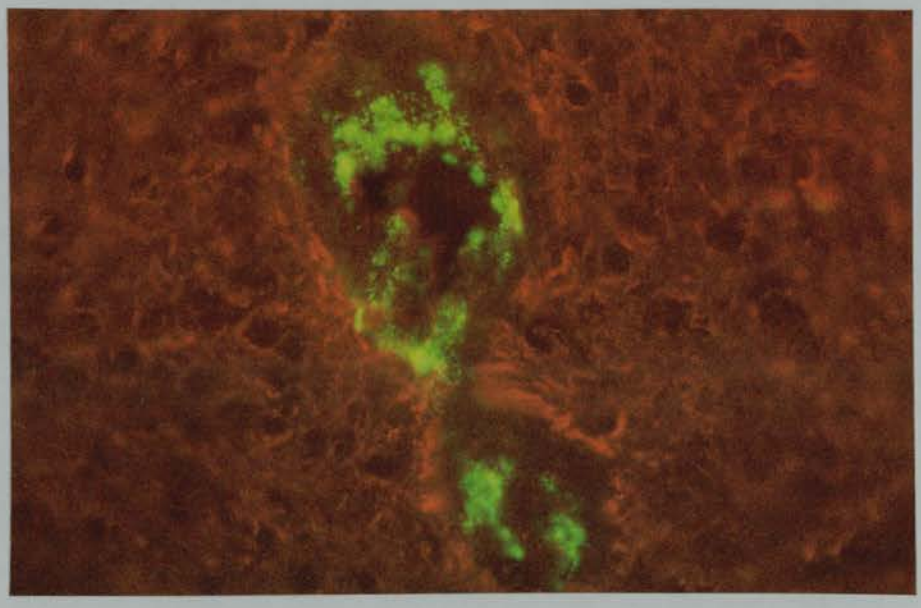
In 17 Grade IV and both Grade III astrocytomas (86% of high grade tumours) vascular staining for t-PA was strong and uniform and particularly easy to see in the hyperplastic endothelial cells of proliferating glomeruloid clusters of vessels (fig.3.12). In two Grade IV astrocytomas strongly and weakly stained vessels co-existed, and in one Grade IV tumour only occasional vessels stained.
In the cortical specimen adjacent to a Grade III astrocytoma the majority of vessels stained strongly although they were not obviously hyperplastic (fig.3.13); in contrast the specimen of normal cortex showed generally very weak staining in numerous small vessels although stronger reaction could be detected in the few larger vessels (fig.3.14).

Perivascular structures.

In larger clusters of tumour vessels a pale halo of FN+ tissue could be seen (fig.3.15) around individual vessels. The interposition of cell processes into this zone has already been commented on. This area was consistently F8RAg-.

Fig.3.11 Double exposure demonstrating t-PA (green), which is predominantly cytoplasmic, in the lining endothelium of small blood vessels, distinct from GFAP (orange) containing tumour cells. High grade astrocytoma, cryostat section, monoclonal anti-t-PA, polyclonal anti-GFAP, x660

Fig.3.12 Two photomicrographs of the same field showing a cluster of proliferating blood vessels in a glioblastoma multiforme staining with UEl (left), and anti-t-PA (right). Note exact correspondence of the two staining patterns which show restriction of reactivity to endothelial cells. Cryostat section, direct UEl, monoclonal anti-t-PA, x660



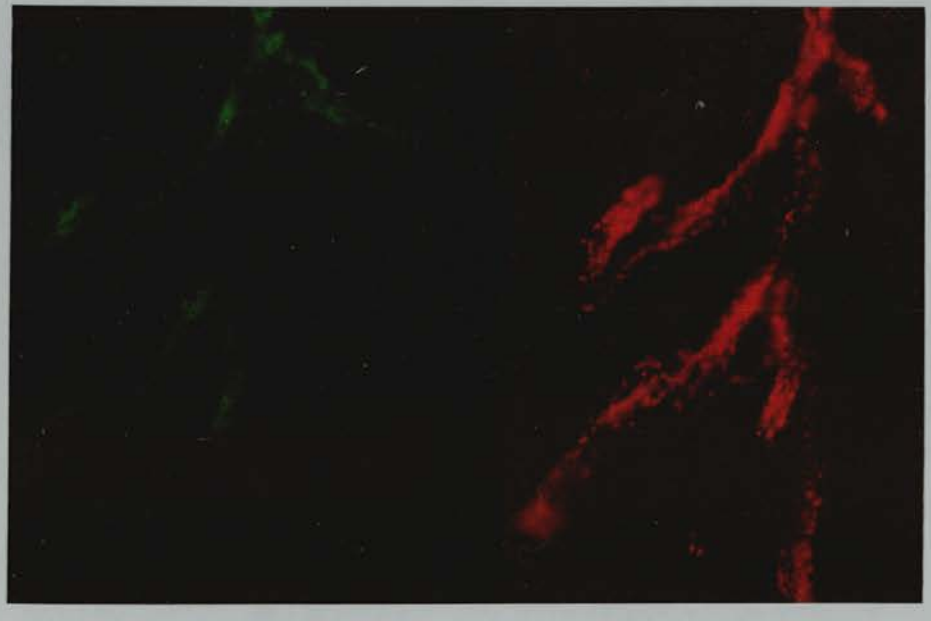
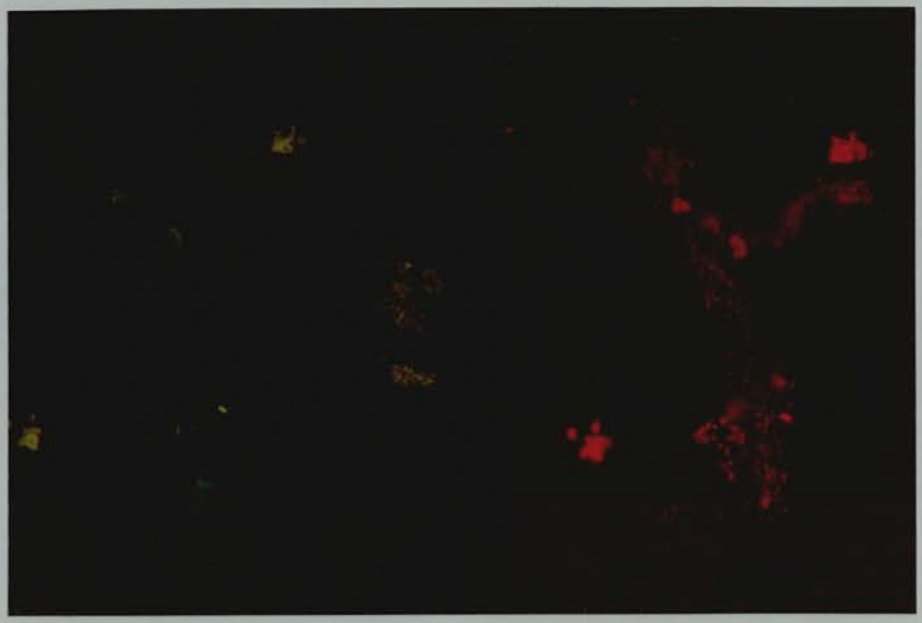
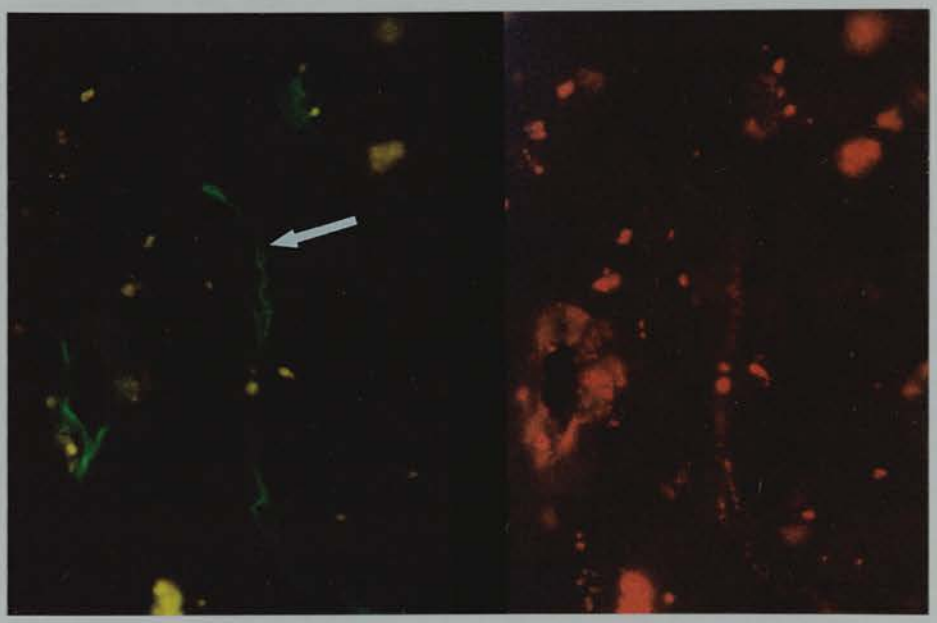


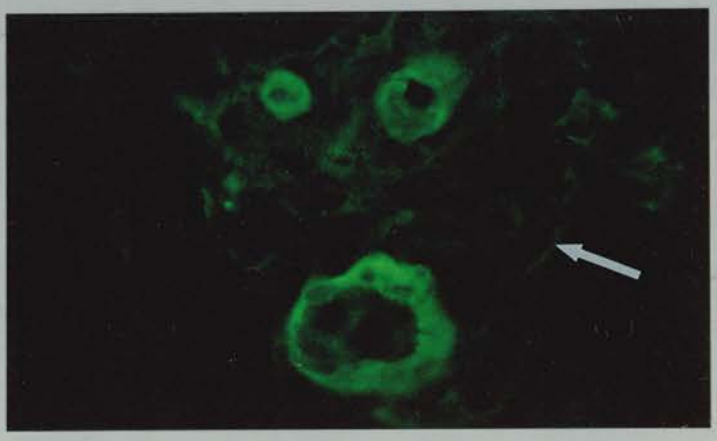
Fig.3.13 Vessels from brain adjacent to an infiltrating grade III astrocytoma show uniform staining with UEl (left) and strong granular staining for t-PA (right). As in tumour vessels (3.12) the two staining patterns correspond. Cryostat section, direct UEl, monoclonal anti-t-PA, x660

Fig.3.14 Large and small vessels from normal brain show similar degrees of staining with UEl (left) but t-PA (right) is less obvious in the small vessel (arrow) in the centre of the field. Cryostat section, direct UEl, monoclonal anti-t-PA, x660

Fig.3.15 Endothelium lining small vessels shows strong fibronectin staining, but a weaker halo can be discerned in the surrounding cuff of mesenchyme (arrow). High grade astrocytoma, cryostat section, monoclonal anti-FN, x520







In three tumours in which a desmoplastic reaction was identified the perivascular zone was greatly expanded and stained much more strongly for FN. The degree of interposition by tumour cell processes was far less and islands of FN- parenchyma were separated by strongly stained mesenchymal zones. In these areas however F8RAg expression was restricted to the cells lining vessels and there was no evidence that they formed a component of the desmoplastic tissue.

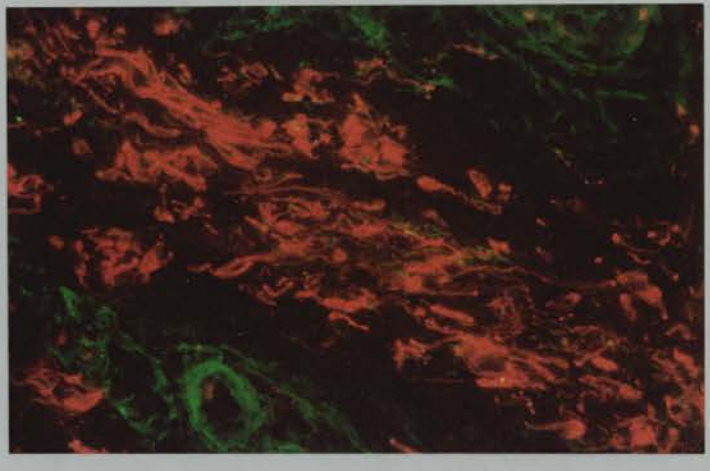
The most striking feature of these three tumours was the strong positivity for desmin (fig.3.16) which contrasted with the inconstant staining seen in non-desmoplastic tumours. It was not possible to identify an increase in vimentin or GFAP staining in these zones since both intermediate filaments were present in considerable amounts in most tumours examined (see above).

Low grade astrocytomas

Parenchyma

All these tumours showed GFAP+ cell bodies and processes and vimentin positivity was more obvious in cell processes. Interposition of cell processes into perivascular areas was limited to the gemistocytic tumour, but this may have been a reflection of the paucity of vascular structures in most tumours of this grade. None of the GFAP+ cells stained for either FN or t-PA.

Fig.3.16 Double exposure of a desmoplastic glioblastoma showing intense staining of tumour parenchyma for desmin (orange) contrasted with the vascular and fibroblastic elements which stain for fibronectin (green). Note the relatively small degree of interposition of tumour processes into the desmoplastic areas. Cryostat section, polyclonal anti-FN, monoclonal anti-desmin, x520



Low grade astrocytomas (cont)

Endothelium

In low grade tumours the appearances of the vasculature more closely resembled that of normal brain than that of the malignant tumours, in that FN staining was generally weaker and in one case was confined to the luminal surface of vessels. F8RAg was likewise more likely to be restricted to the luminal surface but this was not a consistent finding and some vessels showed the patterns described above of strong staining for fibronectin and diffuse cytoplasmic F8RAg. UEl positivity was identifiable on the surface of all endothelial cells but vimentin positivity was more frequent than in malignant lesions. In contrast to the findings in high grade tumours staining for t-PA was absent in three (fig.3.17) and only barely detectable in the gemistocytic tumour. The difference between the proportion of high grade tumours (86%) and of low grade tumours (0%) showing strong vascular staining was highly significant (95% Confidence Limits 72%, 100%; $p=0.003$, X^2 test).

Perivascular structures

These were poorly developed in the low grade tumours; although in two cases, the gemistocytic lesion and one other, there was a defined zone of strong staining for fibronectin surrounding the endothelial layer, in the other two lesions this area was represented only by a halo which stained faintly for FN.

Fig.3.17 A thin walled blood vessel from a low grade cerebral astrocytoma shows defined staining of the endothelium with UEl (left) but no corresponding staining for t-PA (right). Compare with 3.11 to 3.14. Cryostat section, direct UEl, monoclonal anti-t-PA, x660

Other tumours

Staining in these was limited to UEl, F8RAg and t-PA.

UEl positivity was restricted to endothelium in all cases except the three metastases (see below) which showed strong staining of the cell surface of the majority of tumour cells, and two meningiomas which showed focal positive tumour cell staining. F8RAg staining was entirely restricted to the surface and cytoplasm of vascular endothelium.

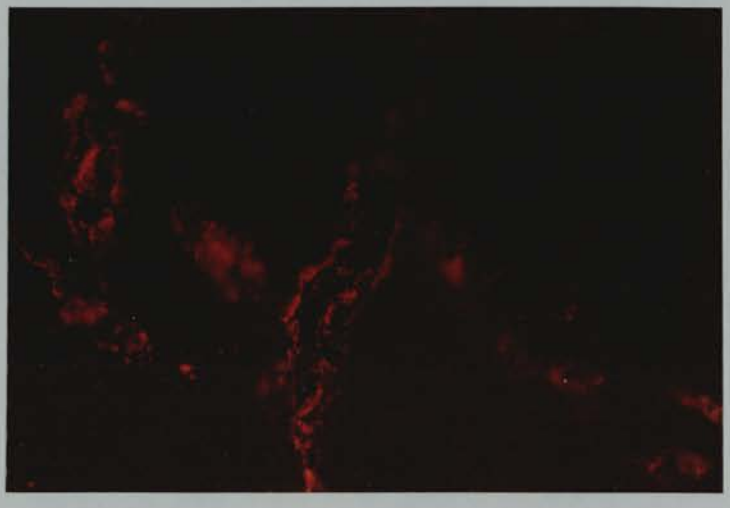
t-PA staining

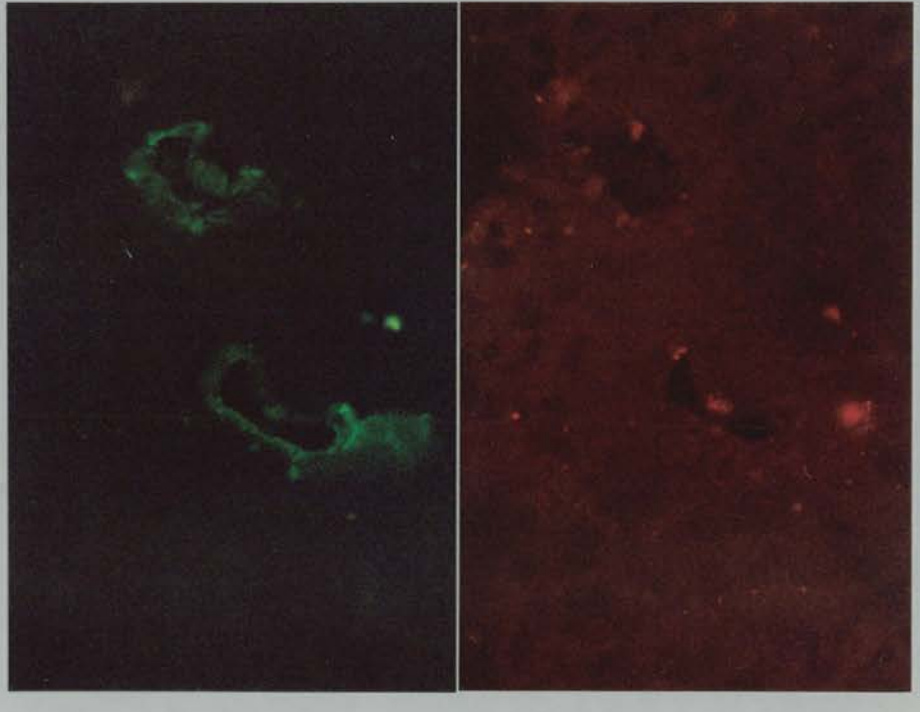
Other gliomas: Uniform strong endothelial staining for t-PA was seen in the ganglioglioma and one choroid plexus papilloma (fig.3.18); mixed strong and weak endothelial staining was seen in the astroblastoma, the ependymoma (fig.3.19) and one choroid plexus papilloma; the oligodendroglioma showed uniformly weak reaction.

In one choroid plexus papilloma and the oligodendroglioma scattered single cells, similar in size and shape to those seen in a few malignant gliomas, showed strong positive staining; in the oligodendroglioma these cells were restricted to an area in which macrophages were present, and in the choroid plexus papilloma the pattern of staining also suggested that non-tumour cells were reacting.

Fig.3.18 Vessels from a choroid plexus papilloma show granular endothelial staining for t-PA. Cryostat section, monoclonal anti-t-PA, x520

Fig.3.19 Vessels in an low grade ependymoma show defined staining of the endothelium with UEl (left) but no corresponding staining for t-PA (right). Cryostat section, direct UEl, monoclonal anti-t-PA, x520





t-PA staining (cont)

Meningiomas: two of these showed no reaction for t-PA and one showed a few faintly stained vessels. The latter lesion also showed focal staining of tumour cells around psammoma bodies.

Metastases: two showed strong endothelial staining for t-PA although in one (an adenocarcinoma) the vessels showed little UEl positivity; a similarly weak reaction for UEl was seen in the third, completely anaplastic, tumour with very weak staining for t-PA. By contrast tumour cells in all three showed strong uniform surface staining with UEl but no t-PA reaction (fig.3.20).

Cell culture preparations

In all these t-PA staining was weak, although above the background level; it did not however compare in intensity with that observed in the endothelium in tissue sections. In the explant specimens a typical pattern of growth was observed: GFAP+ cells comprised the explant with a population of smaller flattened GFAP- cells growing from one edge. As in the cultures described in detail in Chapter 2 the GFAP- cells corresponded morphologically to FN+ cells. GFAP+ cells showed no t-PA staining but FN+ cells showed a weak positive reaction (fig.3.21).

As expected the passaged cultures consisted largely of FN+ cells with only occasional residual GFAP+ cells. There was weak co-expression of t-PA on FN+ cells but not on GFAP+ cells (fig.3.22).

Fig.3.20 Two photomicrographs of the same field of an anaplastic metastatic carcinoma show that t-PA is focally present in vascular endothelium (left) but not in tumour cells, whereas UEl (right) stains tumour cells but not the endothelium. Cryostat section, direct UEl, monoclonal anti-t-PA, x520

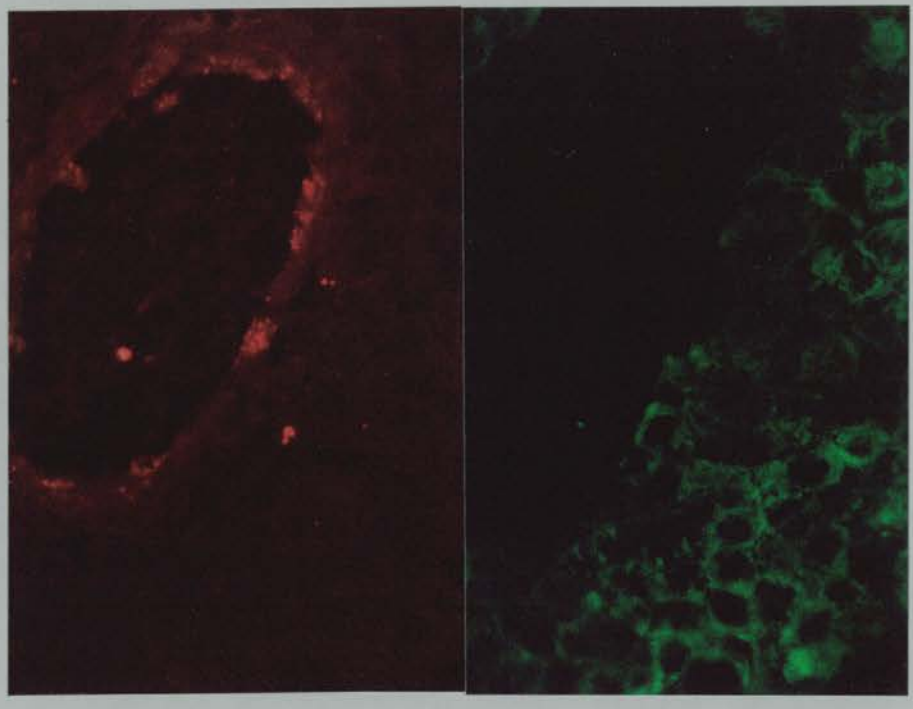
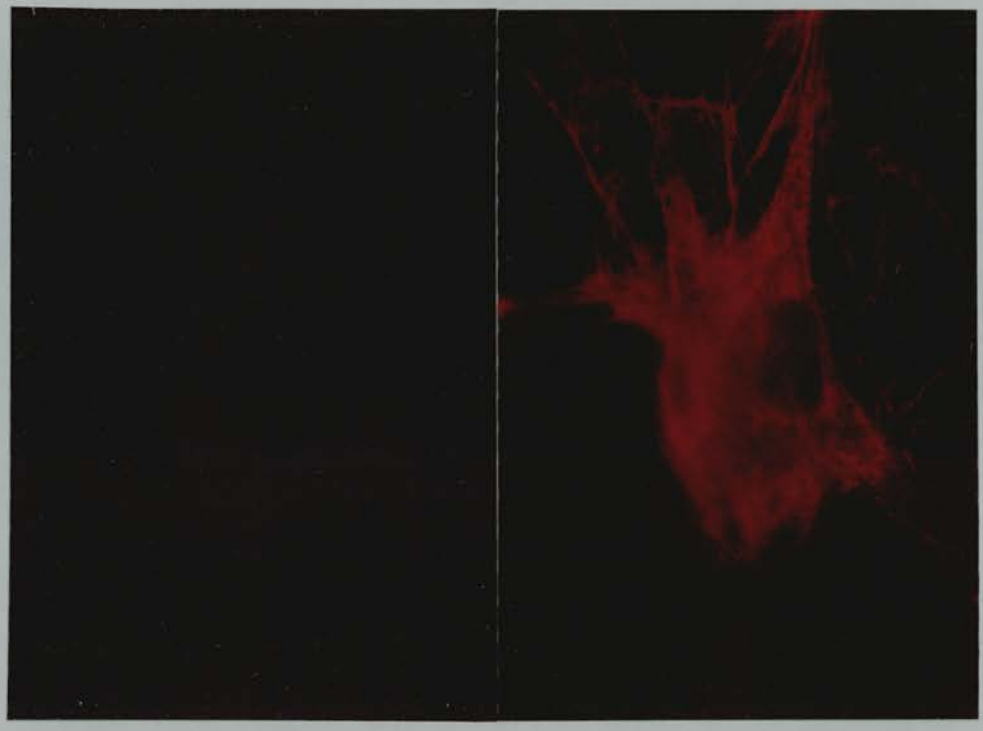
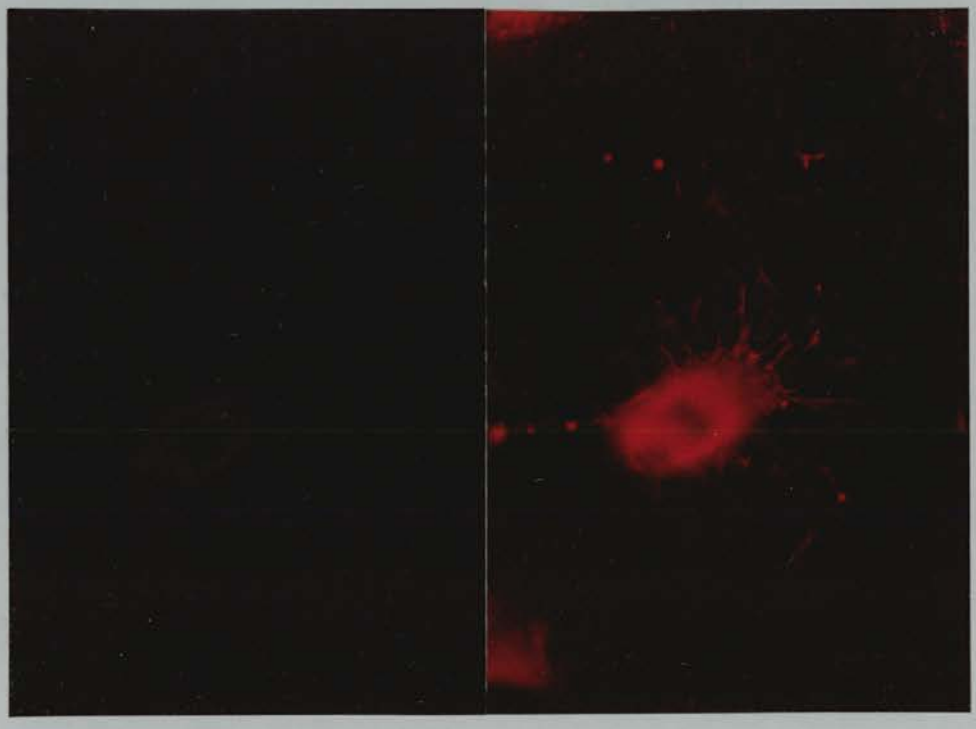


Fig.3.21 Weak t-PA reactivity (left) in cells from a high grade astrocytoma after 28 days in vitro is restricted to FN+ FA cells (right). Polyclonal anti-FN (orange), monoclonal anti-t-PA (green), x520

Fig.3.22 t-PA reactivity (left) in first passage cells from a high grade astrocytoma after 28 days in vitro is restricted to FN+ FA cells (right). Polyclonal anti-FN (orange), monoclonal anti-t-PA (green), x260





DISCUSSION

The co-distribution of the intermediate filaments GFAP and vimentin in the parenchymal cells of the astroglial tumours irrespective of grade is in accord with the findings of previous studies (Herpers et al, 1986; Schiffer et al, 1986; Yung et al, 1985) although a greater degree of cell body staining has been found using an immunoperoxidase technique (Schiffer et al, 1986).

The finding of occasional cells staining for desmin was to be expected given the finding of desmin in normal astrocytes by Dahl and co-workers (Bignami et al, 1985; Dahl et al, 1982; Dahl et al, 1986) but the high levels of expression in the cells of desmoplastic tumours requires some explanation. Given the comments made earlier about the differing specificities of monoclonal antibodies to desmin and the possibility that desmin from different sources may differ antigenically one should concentrate on the distinctive pattern of expression in this group of tumours rather than the specific nature of the intermediate filament. The stimulus to desmoplasia in glial tumours is not always clear but it may be found when glial tumours infiltrate the meninges, or, in a few cases, may develop from what appears to be an essentially perivascular fibroblastic proliferation. The effect on GFAP expression of the proximity to mesenchymal elements has already been referred to; the finding of greater

staining for GFAP than for vimentin in cell processes interposed into perivascular structures and the expression of desmin in desmoplastic lesions could be seen as further examples of such an effect. The alternative explanation that those tumours that express desmin are also the source of a stimulus to fibroblast proliferation remains possible but seems less likely. In vitro studies of cells of known glial nature and the effect on intermediate filament content of exposure to cellular and matriceal components of mesenchyme might provide further insights into this phenomenon.

The differential staining reactions of the vascular endothelium for UEl and F8RAg in all the primary tumours studied showed that such structures are more consistently stained by Ulex lectin than by anti-F8RAg, especially in smaller vessels. This observation confirms the greater sensitivity of UEl for identifying vascular endothelial cells in a variety of tissues including skin and cerebellum (Holthofer et al, 1982), thyroid carcinoma (Stephenson et al, 1986), benign and malignant vascular neoplasia (Leader et al, 1986; Little et al,1986), and cerebral tumours (Bohling et al, 1983; Slowik et al, 1985; Weber et al, 1985).

The tendency for F8RAg in astroglial tumours to be restricted to the luminal surface in larger, apparently non-proliferating, vessels and to show cytoplasmic staining more frequently in the vessels of malignant

lesions may reflect the greater synthesis of F8RAg that has been postulated to occur in proliferating vessels (Miyagami et al, 1987). The latter study found ultrastructural localisation of F8RAg in dilated cisternae of the endoplasmic reticulum in vessels from six out of seven malignant tumours but only one of five benign lesions.

The staining of non-endothelial cells by UEl has been described previously. Normal squamous epithelium in the skin (Holthofer et al, 1982), squamous carcinoma (Leader et al, 1986; Weber et al, 1985) and carcinomas of prostate, large bowel and endometrium have all been found to stain (Leader et al, 1986) but without any consistency. Although this study did not set out to examine the value of UEl reactivity in the diagnosis of tumours the pattern observed in the three metastases is of interest in the light of the observation that staining of breast carcinomas with UEl correlates with outcome (Fenlon et al, 1987) and might repay further study using both cryostat sections and paraffin processed tissue.

With one exception (Kennedy et al, 1987) previous reports of FN localisation in human gliomas have indicated that it is restricted to vascular or mesenchymal structures (Chronwall et al, 1983; Kochi et al, 1983; Paetau et al, 1980; Schiffer et al, 1984). This was the case in all the astroglial lesions in the present study, FN being

restricted to endothelial cells, identified by UEl staining, or to perivascular zones corresponding to areas of vascular or fibroblastic proliferation. The identity of the small number of FN+/GFAP- cells is not obvious. They could be macrophages which have been reported to secrete FN (Rennard et al, 1981) or represent tangentially cut small vessels. The possibility that they represent a sub population of neoplastic glial cells cannot be completely ruled out but seems unlikely on morphological grounds. It is noteworthy that no cells were seen that co-expressed GFAP and FN. The greater FN staining that was observed in vessels in malignant astrocytic tumours when compared to those in normal brain and low grade astrocytomas is in keeping with the known increase in local FN production by proliferating vessels (Clark et al, 1982). The associated observation that there seemed to be less vimentin expression in putatively proliferating endothelium is of interest; it may point to there being a functional association between cell mobility and intermediate filament expression as has already been suggested to exist in glial cells (Duffy et al, 1982).

The monoclonal antibody used to demonstrate t-PA in this study was raised against human melanoma t-PA which has a MW of approx 72kD (Rijken et al, 1981) and appears to be similar to, if not identical with, uterine t-PA which itself appears to be closely related to vessel-associated PA (Ogston, 1983; Rijken et al, 1980). In the present

study it would seem from the nitrocellulose blot that, under the conditions of extraction used, the majority of t-PA in the sample glioblastoma was in the form of two subunits with molecular weights 41 and 31kD, corresponding to previous published findings and a parent molecular weight of 72kD. The weakness of the reaction at 72kD would be in keeping with this interpretation. The nature of the substance reacting at 55kD is not clear; its molecular weight would be consistent with its being a species of t-PA but further studies would be needed to confirm this.

This study shows minimal tumour cell positivity for t-PA in a wide range of human nervous system tumours which is in sharp contrast to the predominant localisation in endothelium. The pattern of cellular distribution also differs, with granular/punctate positivity in endothelium and uniform surface reactivity in the few parenchymal cells that reacted. These findings are consistent with some of the activity being lysosomal in endothelial cells, as found in other species and tissues (Ali et al, 1965), and the finding that in-vitro tumour cell PA is membrane associated (Quigley, 1976).

The fact that only one Grade IV astrocytoma showed tumour cell positivity may reflect the production of cell-associated t-PA in a form not recognised by the antibody used, or may indicate that the bulk of activity measurable in wet tissue is in fact derived from vascular

endothelium. The staining of cells that could have been macrophages in two tumours would accord with the observation that, with appropriate stimulation, PA activity can be induced (Unkeless et al, 1974) although the number of such cells that express t-PA is small when compared to the numbers found with more specific macrophage markers (see Chapter 4).

The finding that t-PA is largely localised in the endothelium of malignant gliomas and some non malignant tumours is not totally unexpected but it highlights the danger of interpreting in-vitro results without knowledge of tissue localisation. From these results it will be apparent that biochemical studies of t-PA levels in tumour specimens will have to take account of tumour vascularity or else demonstrate clearly that the t-PA under study is not localised in vessels.

The degree of staining observed in the cell culture preparations bore no comparison with the intensity of staining seen in tissue sections. Sections stained for t-PA after fixation with ethanol/ether showed no discernible difference from those fixed with acetone confirming that the poor cellular staining was not the result of fixation differences. It is possible that the discrepancies reflect the fact that in vitro most t-PA is secreted into the medium where it will be measureable by its fibrinolytic action (Frame et al, 1984; Freshney et

al, 1985; Hince et al, 1980; McLean et al, 1986) rather than retained in the cell cytoplasm where it would be identifiable immunohistochemically. Endothelial cells in vitro have been found to synthesise an inhibitor of t-PA in vitro as well as the active protease and it is conceivable that such a product could bind to those sites on t-PA that were recognised by the ESP6 antibody.

Whether the form of t-PA observed in this study has a role in nervous system tumour cell invasion is questionable. Identification of other immunologically distinct PAs, such as those studied in colonic carcinomas (Kohga et al, 1985), might reveal secretion by tumour parenchymal cells in gliomas. The finding that t-PA was present in brain vessels adjacent to tumour indicates that its production is an intrinsic property of endothelium which is enhanced when it is stimulated to proliferate by tumour angiogenesis factors. A purified Fibroblast Growth Factor-like substance derived from a human hepatoma cell line has recently been shown to promote angiogenesis in vivo and induce PA activity, enhance DNA synthesis and promote motility in confluent bovine endothelial cells (Presta et al, 1986). Lymphokines have also been shown to have a similar inductive effect on endothelium (Tiku et al, 1985).

These observations provide support for the view that the phenotypically transformed t-PA and FN producing cells derived in-vitro from human malignant gliomas are of vascular origin and are responding to factors produced by neoplastic glial cells (Franks et al, 1986; Jacobsen et al, 1987; McKeever et al, 1987; Manoury, 1977; Rutka et al, 1987a). Cells derived in vitro from human gliomas have been found to secrete an Endothelial Cell Growth Factor-like polypeptide (ECGF) (Libermann et al, 1987) and, more significantly, to possess specific receptors for ECGF. Whilst this may be interpreted variously as a non-specific mechanism for autocrine growth stimulation in neoplastic cells or as a possible mechanism whereby new vessel formation is induced by malignant gliomas, it could be interpreted as evidence for an endothelial origin for the cells concerned. Clearly a study of receptor expression and growth factor production by endothelium, both neoplastic, reactive and normal, from other tissues and the response of cultured cells to glioma-derived factors is required to address this problem in more detail.

The failure of the vessels of the majority of low grade astrocytomas to show t-PA activity raises the possibility that this phenomenon could be utilised in grading of these tumours although this would need examination of larger numbers of cases and of intermediate forms. Vascularity of malignant gliomas, and in particular aberrant vascular

proliferation, has been found to correlate with a poorer prognosis (Cohadon et al, 1985; Fulling et al, 1985) and there might be profit in attempting to correlate t-PA activity with outcome. It should however be stressed that any correlation that was found would be likely to simply reflect the greater degree of new vessel formation that occurs in malignant tumours. Some indirect support for a correlation comes from the finding that the only gemistocytic tumour in this series showed vascular staining for t-PA which resembled that of the majority of high grade tumours despite its lack of histological malignancy; gemistocytic tumours have been held to have a poorer prognosis with a high conversion rate to glioblastomas (Rubinstein, 1972; Schiffer et al, 1988). The important role of the extra protease and FN produced by activated endothelial cells in the process of angiogenesis is underlined by the observed pattern of staining for t-PA and FN in the vessels of high grade gliomas.

Conclusions from in vivo studies

This section of the work has failed to identify cells in the parenchyma of astroglial tumours that show the phenotypic characteristics of the FN+ FA cells derived in vitro from such tumours. With the exception of one grade IV tumour, t-PA expressing cells were restricted to vascular endothelium where they also expressed FN. The possibility that cells of vascular origin, stimulated by factors present in glial tumours, may be capable of overgrowth in culture with phenotypic features interpreted as neoplastic therefore receives support.

The exuberance of the proliferative vascular response in routine sections from many glioblastomas suggests that the component cells of cerebral vessels differ in their responsiveness or are subject to a stimulus that differs at least quantitatively from that provided by other (non-glial) tumours. This would appear to reflect the greater angiogenic stimulus provided by malignant glial cells (Freshney et al, 1985) and cells subject to such a stimulus in vivo might be capable of preferential survival in culture with the phenotypic properties described. Such cells are not restricted to cultures from malignant tumours, and their appearance in cultures from some low grade lesions suggests that a stimulus deriving from malignant glial cells may not be the only factor involved.

This prompts consideration of other possible sources of angiogenic stimuli in astroglial tumours. Lymphokines can induce endothelial t-PA (Tiku et al, 1985) and lymphocytes (Auerbach et al, 1979) and macrophages (Leibovich et al, 1987; Martin et al, 1981; Polverini et al, 1984), either independently or in concert, can provide an angiogenic stimulus. The possibility that such cells might have a bearing on the isolation in vitro of altered vasogenic cells led to a consideration of the cellular immune response to glial tumours.

CHAPTER 4. INTER-RELATIONSHIPS BETWEEN CELLS OF THE IMMUNE SYSTEM, TUMOUR CELL PROLIFERATION AND GROWTH FACTOR RECEPTOR EXPRESSION.

INTRODUCTION

Interest in the body's immune response to glial tumours has derived its impetus from the hope that a natural response to abnormal cells might be exploited therapeutically. Example of such studies have included the effect of the lymphokine leukoregulin on human glioma cells in-vitro (Merchant et al, 1986a) and the potential use of a monoclonal antibody against glioma cells that was also able to complex with liposomes which could deliver drugs directly to tumour cells (Yoshida et al, 1987). Comparable in-vivo work has examined the use of a monoclonal antibody to inhibit growth of experimental grafts of a rat glioma (Stavrou et al, 1986), the direct injection of stimulated autologous lymphocytes into tumours (Ingram et al, 1987) and the use of systemic interferon in humans with malignant gliomas (Boethius et al, 1983). In the latter study, in which only one patient appeared to benefit and even then not clearly as a result of therapy, it was noteworthy that there was no correspondence between the response of tumour cells to the agent in vitro and the response of the same tumour in vivo. Intratumoural antigenic heterogeneity and the rarity of tumour-specific antigens that are not also expressed by some normal cells have been identified as major

difficulties in this approach to therapy (Colnaghi et al, 1988).

Detailed examination of the immune response to glial tumours has revealed complex cellular interactions. Among the possibilities that have to be considered are not only i) an inhibitory effect of immune cells on tumour cells but also ii) an inhibitory effect of tumour cells on immune cell function, iii) the production of specific factors by glioma cells that protect against an immune response, iv) the enhancement of an immune reaction by tumour cells and v) a stimulatory effect of the immune response on tumour cells.

i) Do immune cells inhibit tumour cell growth?

Peripheral circulating lymphocytes taken post-operatively from patients with malignant glial tumours can be induced in vitro to kill cells derived from the tumours only when subjected to prior stimulation with Interleukin-2 (IL-2) (Jacobs et al, 1986). It is of note that such killing was not restricted to autologous cells suggesting that the mechanisms may be non-specific although initiated by an immune interaction. This interpretation is supported by the observation that non-specifically activated lymphocytes release factors in vitro that inhibit DNA and RNA synthesis in glioblastoma cell lines irrespective of the patient's immune status or even whether or not a glial tumour was present (Merchant et al, 1986). In the latter

study the factors that were inhibitory to neoplastic glial cells also appeared to both stimulate and induce differentiation in fetal rat glioblasts, emphasising that the observed effect of any interactions between immune and glial cells will also depend on the susceptibility of any target cells to any factors that are produced by such interactions.

ii) Can tumour cells have an inhibitory effect on immune cell function?

Studies that have pursued the question of why autologous lymphocytes from patients with malignant gliomas frequently fail to kill cells from such tumours in-vitro (Gately et al, 1982) have directed attention to inhibitory factors produced by the tumour cells. The lymphocytes appear to have a reduced proliferative response to stimuli that would normally evoke proliferation (Elliot et al, 1984; Miescher et al, 1988); such proliferation as does occur is associated with lower than expected levels of production of the lymphokine IL-2 (Elliot et al, 1984; Elliot et al, 1987; Elliot et al, 1987a) and lymphocytes also bear fewer surface receptors for IL-2 (Elliot et al, 1987). A number of factors produced by glioblastoma cells have been identified as being responsible for these effects including Prostaglandin E2 (Lauro et al, 1986) and larger molecules with molecular weights variously reported as 97 (Fontana et al, 1985), 25 (Roszman et al, 1987) and 12.5kD (Wrann et al, 1987). The latter factor showed

homology with 8 out of the first 20 amino acids in Transforming Growth Factor (TGF) and may be the active component in the larger molecular weight fractions reported. Studies of inhibitory factors produced by non-glial tumours have demonstrated a close homology of a 19kD protein with the p15E fragment of a retrovirus envelope (Cianciolo et al, 1986) raising the possibility that an oncogene is involved. Further speculation along similar lines (Normann, 1985) has suggested that the immunosuppression observed in relation to certain tumours may be the result of the activation (in both immune and neoplastic cells) of mechanisms evolved to reduce the immune response to the developing fetus. Such a process could be locally initiated by macrophages attracted to the tumour and subsequent events are then the inevitable consequence of activation of intrinsic mechanisms in which the tumour is not only the inadvertent initiator but also the unintended beneficiary. Reports of a more generalised impairment of immune function in glioma patients include a degree of cutaneous anergy proportional to tumour grade (Mahaley et al, 1977), the finding of a reduced ratio of helper to suppressor subtypes of peripheral blood T lymphocytes (Bhondeley et al, 1988; Bullard et al, 1986; Uegaki et al, 1988) and reduced lymphocyte mitogen sensitivity (Blom et al, 1985). The influence of therapy in these studies varies and others have attributed the observed changes to secondary effects of disturbances of the brain-pituitary-endocrine axis on immune function

(Uegaki et al, 1988). This is supported by series in which no differences from normal are found except in cases with tumours so located that hypothalamic function might have been interfered with (Palma et al, 1987).

iii) Do glioma cells produce specific factors that protect against an immune response?

The observation that some glial tumours appeared to have very few infiltrating lymphoid cells (in contrast to others which had many) led to the suggestion that sialomucins secreted by the tumour may inhibit the development of a specific immune response to tumour antigens (Ridley et al, 1971; Stavrou et al, 1977). More direct in-vitro analysis demonstrated the production of a hyaluronidase-sensitive coating around some glioma cells which physically interfered with contact by surrounding lymphocytes (Gately et al, 1982). This coating seemed to reduce the capacity of the cells to evoke a non-specific cytolytic response from autologous and allogeneic lymphoid cells. Its secretion could also be stimulated by factors in the supernatant from mixed lymphocyte reactions but glial cells from later passage cultures (Oberc-Greenwood et al, 1986) were no longer capable of its production, possibly because selection of cells had resulted in the loss of genuine glial elements.

iv) Can tumour cells enhance an immune reaction?

Interleukin-1 (IL-1) is secreted by macrophages as part of the response to interactions between macrophages and lymphocytes that follow the presentation of antigen by the former to the latter (Roitt et al, 1985). One of its main functions in this context is to stimulate the proliferation of lymphocytes with immune specificity to the presented antigen. Normal rat (Fontana et al, 1984; Frei et al, 1985; Frei et al, 1986) and neoplastic human astrocytes (Fontana et al, 1984; Fontana et al, 1985) produce interleukins which enhance lectin-induced mitogenesis in lymphoid cells (Fontana et al, 1984) and can directly induce macrophage proliferation (Frei et al, 1986). With the evidence that anti-proliferative factors are produced by astroglial cells the net effect may depend on the balance existing in a tumour between stimulating and inhibiting factors; conflicting findings relating immune status to clinical outcome may reflect the fact that such a balance may change with time thus complicating interpretation.

v) Can an immune response enhance tumour cell growth?

As stated above glioblastoma cells produce interleukins, and both fetal rodent astrocytes (Giulian et al, 1985) and a human glioma cell line have been reported to show a proliferative response to IL-1 (Lachman et al, 1987). There is a considerable body of evidence concerning the capacity of macrophage-produced factors to enhance,

promote or stimulate tumour growth. Although much of this stems from studies of an experimental murine fibrosarcoma (Currie, 1981; Evans, 1977, 1978, 1984; Evans et al, 1984; Evans et al, 1984a) which may have had singular properties and growth requirements, Tumour Necrosis Factor (TNF), which is of macrophage origin, has been shown to act as a growth factor in chronic B-cell malignancies in humans (Cordingley et al, 1988). Macrophages are probably present in varying amounts in most tumours and a 12kD factor has been identified in supernatants from experimental and human tumour cell cultures that is chemo-attractant to macrophages (Botazzi et al, 1983). The finding that embryonic fibroblasts (both human and murine) also produced the factor suggests that it is part of a fundamental process of cellular interaction rather than a phenomenon associated with the neoplastic state. The attraction of macrophages to tumours gains some importance with the suggestion that they may play a role in determining the onset of neovascularisation and thus an accelerated rate of growth (Folkman, 1984). The direct involvement of macrophage-derived factors in angiogenesis has already been mentioned in Chapter 3.

The possibility that neoplastic glial cells may derive a positive stimulus to growth from factors secreted by cells of the immune system receives some support from observations of the expression of certain surface proteins by tumour cells.

The expression of Major Histocompatibility Complex (MHC) Antigens by tumour cells

Some interactions between cells of the immune system are dependent on the presence of a class of surface antigens of the Major Histocompatibility Complex (MHC). The MHC gene is located on chromosome 6 and encodes for a series of surface proteins which in combination are unique to the individual; because of their original discovery in the context of transplant matching using leucocytes these were originally termed Human Leucocyte Antigens (HLA).

The gene coding for these proteins is divided into five regions:

Three of these, A, B and C, code for MHC Class I proteins which are expressed on the surface of all nucleated cells and are concerned with the recognition of self and non-self cells, the latter being either foreign tissues or cells expressing viral antigens during a productive infection.

Region D codes for MHC Class II proteins which are usually only found on the surface of cells which fulfil the role of presenting foreign antigen to cells such as lymphocytes which will react by the development of an appropriate immune response. MHC Class II proteins are found therefore on macrophages, Langerhans cells in the skin and some endothelial cells. The interactions of Class II-expressing cells with potentially responsive cells often result in the secretion of growth factors that stimulate the

reacting cell to proliferate. Three subtypes of D antigens are recognised, namely P Q and R, although they are often collectively referred to as HLA-DR (D-related).
The fifth region codes for some complement components but these are not expressed on the cell surface.

MHC, and other, antigens typically associated with the surface of haemopoietic cells were found to be expressed by cells (assumed to be glial) in sections from gliomas (Budka et al, 1985; Budka et al, 1985a; Chilosi et al, 1986; Motoi et al, 1985), and also by renal (Borowitz et al, 1986) and pulmonary tumour cells (Patterson et al, 1985; Ruff et al, 1984). The authors of such studies saw such expression mainly in terms of a marker of tumour type and as a source of potential confusion if these antigens were to be used as diagnostic markers. In gliomas these positive cells were usually considered to be astroglial in nature, often on the basis of a process-forming morphology, but at the time the extent of macrophage infiltration in gliomas was not generally appreciated (Chilosi et al, 1986).

Expression in vitro of similar antigens by glioma-derived cells or cell lines (Carrel et al, 1982; De Murault et al, 1985; Studer et al, 1985; Wikstrand et al, 1985) was generally found to be heterogeneous with no obvious correlation with morphology. It was suggested that these antigens had a potential value in classification of tumour

type or grade and that they might be of functional importance.

Further study provided evidence that functionally astrocytes might have much in common with macrophages. In the normal brain Class II antigens were found to be only sparsely expressed, mostly on endothelial cells and a few astrocytes or microglia (De Tribolet et al, 1984; Lampson et al, 1986). Astrocytes were found to express not only much more Class II antigen in active chronic multiple sclerosis (Traugott et al, 1988) and around abscesses and metastases (Frank et al, 1986), but rat astrocytes, both normal and neoplastic, were also found to possess phagocytic properties in vitro (Bjerknes et al, 1987). Class II antigen expression could be experimentally induced on astrocytes by measles antigen; macrophage-derived TNF enhanced the response, but was not not capable of eliciting it on its own (Massa et al, 1987).

Class II antigens were found to be inducible by gamma interferon on the surface of cultured fetal (Hirayama et al, 1986; Pulver et al, 1987), adult (Kim et al, 1985) and neoplastic (Piguet et al, 1986; Piguet et al, 1986a; Takiguchi et al, 1985) human astrocytes. This induction seems to be a specific effect involving active transcription of the gene, one section of which appears to be sensitive to interferon (Basta et al, 1987). Not all neoplastic glial cells were found to be sensitive to

interferon, apparently due to a transcriptional block at the level of the HLA-DR gene rather than a lack of interferon receptors (Piguet et al, 1986a). The HLA antigen that is induced on neoplastic glial cells, and that naturally present on melanoma cells (Guerry et al, 1984), is functional and capable of evoking an appropriate lymphocyte response (Takiguchi et al, 1985). Astrocytes have been found to be capable of replacing monocytes in an in-vitro test of lymphocyte mitogen response but interestingly this did not appear to depend on surface Class II antigen expression (Schnyder et al, 1986). The authors of this study suggested that in addition to interleukin-1 (IL-1) neuraminidase may also be involved; they postulated that this, by reducing the high surface negative charge on the lymphocytes, would facilitate cell to cell contacts which, in turn, would enhance cell interactions and thus proliferation. All these studies suggest that the normal astrocyte may serve the function of an antigen-presenting cell in the CNS and that the interactive capabilities of such a cell might be retained following neoplastic transformation.

Immune cell infiltrates in gliomas

Early studies of the lymphocytic infiltrate in gliomas highlighted the often strikingly perivascular location of the infiltrate (Bertrand et al, 1960; Ridley et al, 1971); lymphoid infiltration has been associated with tumour necrosis (Brooks et al, 1978; Ridley et al, 1971) or

(Bertrand et al, 1960; Takeuchi et al, 1976) the presence of gemistocytic cells in the tumour.

Studies relating the degree of lymphoid infiltrate to survival have found some association between survival and a high level of perivascular (Brooks et al, 1978) or interstitial (Palma et al, 1978) lymphoid infiltrate in a small number of high grade gliomas (Brooks et al, 1978; Palma et al, 1978). A relation between the degree of lymphocytic infiltrate and mean survival time was found for a larger series of tumours but the values were found to be of little value for prognosis in an individual patient (Boker et al, 1984). That these could constitute a distinct subgroup is suggested by the apparently contradictory finding that in general cellular infiltrates were higher in high grade than low grade lesions (Bertrand et al, 1960). Published (Palma et al, 1978) and unpublished observations (Cooper MH, 1986) have indicated an inverse association between lymphocytic infiltration and patient age; an apparent effect of the former on survival could therefore be explained by a direct effect of the latter. In studies of the associations between survival and lymphocytic infiltration neither patient age nor the influence of lymphocyte subtypes have been studied in any detail.

A number of studies have been aimed at assessing the pattern of the cellular response to human gliomas, with particular reference to the cell types involved. Although

one group claimed to have identified a relatively high proportion of B cells on the basis of surface immunoglobulin (Yasuda et al, 1983) others emphasised the presence of T cells and the frequent absence of B cells (Hitchcock et al, 1988; Paine et al, 1986; Stavrou et al, 1977; Von Hanwehr et al, 1984). Comparison between the quantitative analyses of T cell subtype are difficult due to the lack of consistency in the methods used to present cell counts, but it is clear that no consistent pattern is found in astroglial tumours. Although T4/T8 ratios were often low, in keeping with a suppressor effect (Hitchcock et al, 1988; Stevens et al, 1988), some tumours showed a predominance of helper cells and others showed no significant lymphoid infiltrate. Although an antibody against Human Natural Killer (HNK) cells had shown apparent reactivity on some tumour cells in astrocytic and oligodendroglial tumours (Motoi et al, 1985) other authors concluded that HNK cells were themselves scarce in gliomas and played little part in the immune response to these tumours (Stevens et al, 1988). Earlier studies assumed the majority of mononuclear cells to be lymphoid but recent studies have noted the high proportion of monocytes in many tumours (Hitchcock et al, 1988; Rossi et al, 1987; Rossi et al, 1988).

Even when a statistical association was found between the level of histological infiltrate and an in vitro measure of lymphocyte function the fact that a number of

individual cases failed to show such an association emphasised the importance of assessing the pattern of cell types as well as the degree of cellular infiltration (Boker et al, 1982).

It would seem from the above considerations that glioma cells are potentially capable of responding to a range of factors produced by cells of the immune system infiltrating the tumour as part of an evoked response. They are however also potentially capable of responding to at least one defined growth factor as judged by their expression of the cell surface receptor to it, namely the Epidermal Growth Factor (EGF).

Epidermal Growth Factor and glial tumours

EGF was first described in 1962 (Cohen, 1962) in an isolate from salivary gland and shown to have mitogenic properties. The receptor for EGF (EGFR) was one of the first human gene products shown to be homologous with the product of an experimental oncogenic virus, the avian erythroblastosis virus (Downward et al, 1984). EGFR is a complex protein encoded for by the erb-B proto-oncogene on chromosome 7 (Waterfield et al, 1983). It possesses an extracellular portion responsible for EGF binding, an intramembrane domain, and an intracellular portion which has tyrosine kinase activity which is thought to be responsible for linking receptor binding to cell division (Thompson et al, 1985). In cells transformed by the

Erythroblastosis virus (Downward et al, 1984) and in malignant human epidermal cells (Ozanne et al, 1985; Ullrich et al, 1984), EGFR is expressed in a truncated form, lacking the extracellular domain, and in this form it appears to act as if EGF has bound to it despite the absence of its external section. Not only is the EGFR in these circumstances abnormal but it is often present in increased amounts either due to amplification of its coding gene, or to increased transcription which may relate to chromosomal translocations that have freed the gene from the influence of its normal regulators (Ozanne et al, 1985; Ullrich et al, 1984). As a result cells show the inappropriate proliferation that is one of the features of the malignant state. The central part played by this mechanism in the transforming process is attested to by the fact that the function of a growth factor that apparently effects cellular transformation in-vitro (Transforming Growth Factor - TGF) is now known to depend on its binding to EGFR although it differs in structure from EGF (DeLarco et al, 1980).

The expression of EGFR on normal glial cells was reported in 1977 (Westermark, 1977) but it was not until seven years later that the receptor was described in gliomas by Libermann and colleagues (Libermann et al, 1984). This group found that the levels of expression varied from tumour to tumour and, while some did show a definite increase in levels, it was not clear whether this

reflected an general increase in expression by neoplastic cells or the growth of a population that naturally expressed higher levels of receptor but were scarce in non neoplastic tissue. They subsequently showed (Libermann et al, 1985, 1985a) that enhanced receptor levels were associated with gene amplification and that up to one third of malignant glial tumours might show such enhanced expression.

Studies on glioblastoma cell lines have yielded more detailed information on EGFR structure and function. The EGFR of gliomas differs from that of neoplastic squamous cells in that it has a higher affinity for EGF and is more heterogeneous in its structure (Westphal et al, 1985). More significantly the receptor seems to be more uncoupled from the normal processes of cell growth inhibition rendering the cells that express it less susceptible to control by cell density (Westphal et al, 1985).

Against this background of the known expression of EGFR and of surface HLA class II proteins by some gliomas, and indirect evidence that immune elements could serve to stimulate tumour growth a study was undertaken to determine the relationship, if any, between tumour antigen expression, immune cell (especially monocyte) infiltration and tumour growth.

MATERIALS AND METHODS

The work described in this section was carried out in three separate phases determined by a combination of tissue and antibody availability. The initial study was aimed at assessing the degree and pattern of MHC Class II antigen (MHCII) expression in a small series of astroglial tumours. Subsequently a larger series was examined and an analysis was carried out of correlations between lymphocyte and macrophage infiltration, MHCII expression and tumour cell proliferation. In the last section the pattern and degree of Epithelial Growth Factor Receptor (EGFR) expression is explored.

The tissue examined in this study derived from diagnostic specimens received in the Neuropathology Laboratory, University of Leeds between 1983 and 1987. Fresh tissue had been frozen in isopentane or directly in liquid nitrogen and examined after storage periods of up to three and a half years. All tissue cultures derived from the material that has been fully described in Chapter 2 and the technical methods used were identical to those detailed in that section.

The methodology for this section is detailed together for convenience. However since the findings in the first study had a bearing on the nature of the next two these results are presented and discussed separately.

Reagents

A panel of antibodies was used to identify MHCII antigens, lymphocytes and their subsets. The specificities of the antibodies used and their dilutions are detailed below. The main lymphocyte markers used were T11 and T28, chosen because they would identify the great majority of lymphocytes infiltrating tumours irrespective of their subset and their state of activation. While this fails to take account of the functional significance of T cell subsets wide variations in the numbers of such cells have been reported in gliomas (Hitchcock et al, 1988; Miescher et al, 1988; Paine et al, 1986; Rossi et al, 1987; Von Hanwehr et al, 1984) and a broader approach was felt necessary to address the question of any relationship between lymphocyte and monocyte infiltration and tumour antigen expression.

In all studies on tissues 8μm cryostat sections were freeze-dried overnight, then fixed for 20 minutes in acetone.

Details of the Monoclonal Antibodies used:

MHC Class II:

Name	Source	Dilution	Identifies
TAL-IB5 (Adams et al, 1983)	ICRF	Neat	HLA DP, DQ, DR
CR3/43 (Falini et al, 1986)	MRC-CG*	1:10	HLA DP, DQ, DR
DA6.231 (Guy et al, 1982)	MRC-CG*	1:10	HLA DP, DQ, DR
DA6.147 (Guy et al, 1982)	MRC-CG*	1:10	HLA DQ, DR
B721	MRC-CG*	Neat	HLA DP
DA6.164 (Krajewski et al, 1985)	MRC-CG*	1:4	HLA DR (except DR7 and weak DR3)
Leu10 (Brodsky, 1984)	Becton-Dickinson	Neat	HLA DP, DQ, DR (not DR3 and DR7)

*A gift from Dr M Steel, MRC Cytogenetics Unit, Edinburgh.

Lymphocytes: all diluted 1:10 except T6 (1:20)

Name	Source	Identifies	Cluster of Differentiation (Bernard et al, 1984)
T28	Unipath	Mature T cells	CD3
T11	Dakopatts	Pan-T cell	CD2

Macrophages: diluted 1:10

Name	Identifies
Dako-macrophage	Human macrophages; antigen unidentified
Dako-p159,95	Macrophages (Schwarting et al, 1985; Falini et al, 1986)

Epithelial Growth Factor Receptor: diluted 1:200

EGFR1 a gift from Dr M Waterfield, ICRF, London (Waterfield et al, 1983)

Proliferating cells: diluted 1:5

Ki67	Dakopatts	recognises nuclear protein in proliferating cells (Gerdes et al, 1984; Gerdes et al, 1985; McGurrin et al, 1987)

Polyclonal antisera

anti-GFAP, anti-Fibronectin and anti-F8RAg all from Dakopatts used at 1:200 dilution

Demonstration of MHC Class II antigens

1. Rehydrate sections in Tris buffer
2. Incubate with primary monoclonal antibody (TAL-IB5, CR3/43, DA6.231, DA6.147, B721, DA6.164, Leu10) at optimum dilution in tris buffer, for 2-3h
3. Rinse in tris buffer then wash for 1 to 5 min in Tris
4. Incubate with 1:50 rabbit-antimouse peroxidase conjugate (diluted in 1:20 human AB serum) for at least 15 min
5. Two 5 min washes in tris buffer.
6. Visualise in 0.005% DAB (3'3' diaminobenzidine tetrahydrochloride monohydrate solution with hydrogen peroxide in tris) for 5-10 min
7. Wash in water
8. Incubate in 0.5% copper sulphate (in 0.9% saline) for 5 min
9. Wash in water
10. Counterstain with methyl green

In sections stained with these antibodies a semi-quantitative assessment (0,+, ++) was made of whether antigen was present on cells identifiable as tumour cells, by virtue of size and process formation, and on smaller process-free cells tentatively identified as lymphocytes. Note was also taken of the pattern of staining with particular reference to perivascular enhancement.

Demonstration of lymphocytes, macrophages and proliferating cells

a) Sections from four grade IV astrocytomas were stained by the double labelling immunofluorescent technique described earlier (pg 39) using a number of monoclonal antibodies against lymphoid (T28, T11), macrophage (Dako-macrophage, Dako-p150,95) and MHCII (CR3/43, DA6.231, DA6.147) antigens, in combination with a polyclonal anti-GFAP antiserum. The distributions of positively stained cells and their coexpression of GFAP and FN were noted.

b) Cultures from five astrocytomas (four grade IV and one low grade) were examined for co-expression of MHCII using the monoclonal antibody DA6.231, and GFAP or fibronectin (FN) using a polyclonal antiserum, with a double immunofluorescent method.
Note was taken of the patterns of coexpression among the different cell types previously recognised in tissue cultures from gliomas.

c) In 29 tumours serial sections were stained with antibodies to identify T lymphocytes (T28, T11), macrophages (Dako macrophage, Dako-p150,95), MHCIIs (DA6.231) and proliferating cells (Ki67) using the indirect immunoperoxidase method described above. These sections were counterstained with haematoxylin and weak

eosin which allowed identification of vascular structures and unstained cells in all sections.

It was intended initially to assess the relative density of cells in perivascular and interstitial areas of tumours. Perivascular cells included all those within 30μm of a vessel or within a cuff of infiltrating cells, and an index was derived by calculating the proportion of all stained to all unstained cells. It soon became apparent that the results were heavily affected by the type of vessel and whether cells aggregated in a perivascular cuff or not. Thus a mature thin walled vessel with a focal cuff would register a very high index whereas a proliferating vessel with relatively more cellular elements would register a lower index despite heavy interstitial infiltration.

This method of assessment was therefore abandoned in favour of counts of interstitial cell density which could be argued to more closely reflect the likelihood of tumour cell/immune cell interactions.

Counts were therefore made of all stained and unstained cells in ten 0.09mm^2 fields (using a 10 by 10 eyepiece grid and a 25x objective) of interstitial tumour. The latter was defined as all tissue further than 30μm from a vascular structure. Positively stained cells were expressed as a percentage of the total.

Since the intention was to make comparisons between tumours such a methodology was considered preferable to

the use of absolute counts per unit area which would be affected by tissue distortions that would differ from case to case and even from section to section. It is not unreasonable to assume that any such effect will be the same on all cells in a section irrespective of their staining reaction.

To assess the degree of cell proliferation in Ki67 preparations counts were made of stained and unstained cells in successive, different fields of 0.0361 sq mm (using a 10 by 10 eyepiece grid and a 40x objective) until a minimum of 1000 cells had been counted. The proliferative index was expressed as a percentage of all cells that stained positively. To avoid bias the Ki67 preparations were stained on a separate occasion to the remainder and analysed as a batch without knowledge of the results of the previous measurements.

Based on the frequency distribution of values for Ki67 staining in gliomas, three grades were constructed delineating respectively the bottom 33% (0 to 33rd centile), the middle 33% (34th to 66th centile) and the top 33% (67th to 100th centile). These grades were used in a comparative analysis with the grade of EGFR1 staining.

Demonstration of Epithelial Growth Factor Receptor

Double immunofluorescence utilising the antibody EGFR1 in combination with a polyclonal antibody against GFAP was used to study expression of EGFR on parenchymal cells in tumour sections. In tissue culture preparations EGFR1 was used in combination with polyclonal antisera against GFAP, FN and F8RAg.

The double staining technique used has already been detailed in Chapters 2 and 3.

In tissue sections and culture preparations EGFR1 staining was assessed semiquantitatively (0 negative, + focal weak, ++ focal strong, +++ uniform strong). In culture preparations co-expression of other antigens was noted.

Statistical analyses

Correlations between the percentage of cells staining positively with each method were carried out using Spearman's rank correlation with two tailed significance testing. Differences between the findings in high and low grade astrocytomas were determined by a Mann Whitney U test with two tailed significance testing. The differences between the two measures of macrophages and T cells were assessed by 95% limits of difference for each pair of markers, and a paired t significance test (Bland et al, 1986).

Possible associations between EGFR1 grade and the level of Ki67 staining were studied with a Kruskal-Wallis one-way analysis of variance (Siegel, 1956).

A. THE FREQUENCY, DISTRIBUTION AND SOURCE OF MHC CLASS II ANTIGEN STAINING IN GLIOMAS.

MATERIALS

Tissue from 12 astrocytomas (nine Grade IV, two Grade III and one Grade II) was used. In addition 12 primary cultures (three explant and nine dispersed cell) from five high grade astrocytomas were studied, all after 16 days or less in vitro. Earlier experiment had showed that MHCII did not survive the methanol fixation that had been standard for cell cultures in the initial studies and in consequence these and all subsequent cultures were fixed with ethanol/ether as described in Chapter 2.

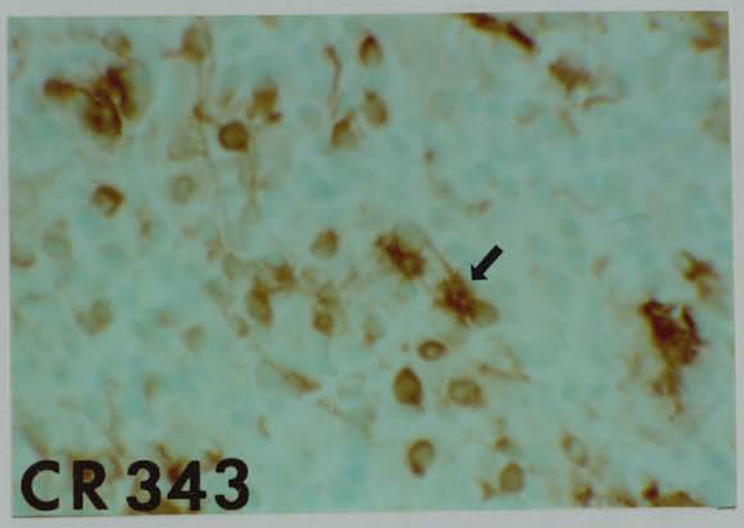
INITIAL RESULTS

In vivo

Peroxidase stained sections from all 12 tumours showed some reactivity with some or all of the monoclonal antibodies used. Positive staining of discrete round cells in the tumour interstitium or around blood vessels was a feature of all sections (fig.4.1). The intensity of this infiltrate varied from case to case but was not quantified. Distinct from this staining of what were considered to be non-parenchymal cells was reaction related to larger process-forming cells (fig.4.2) which in a number of cases showed a clear enhancement in tumour tissue around blood vessels (figs.4.3 & 4.4).

Fig.4.1 Staining for MHCII is seen on small and large cells, some of the latter obviously process-bearing and morphologically resembling astrocytes (arrow). High grade astrocytoma, cryostat section, CR3/43 peroxidase, x432

Fig.4.2 Much of the MHCII reaction in this section is on cell processes; the process-bearing nature and astrocytic morphology of positively stained cells is apparent. High grade astrocytoma, cryostat section, CR3/43 peroxidase, x432


CR 343

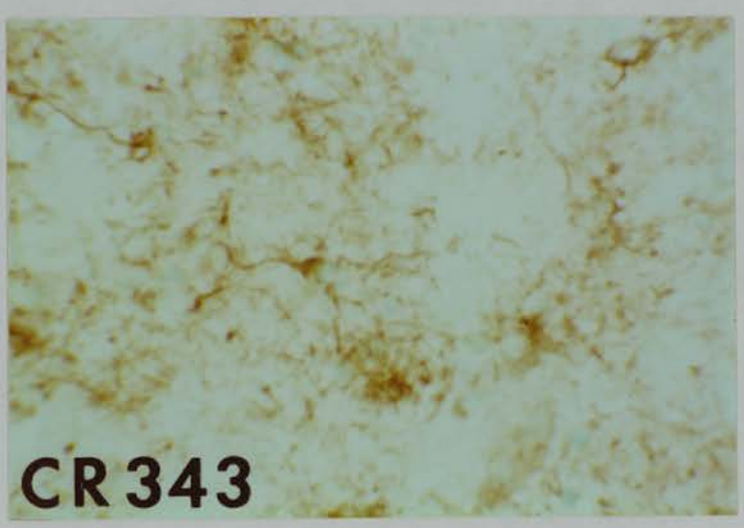
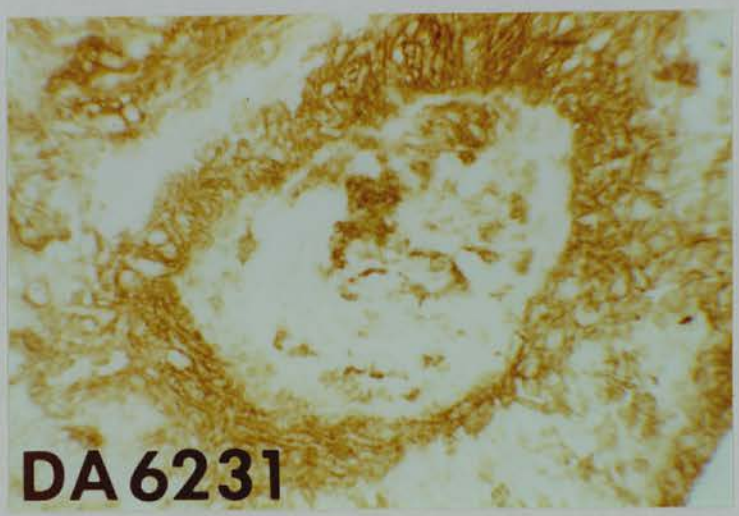
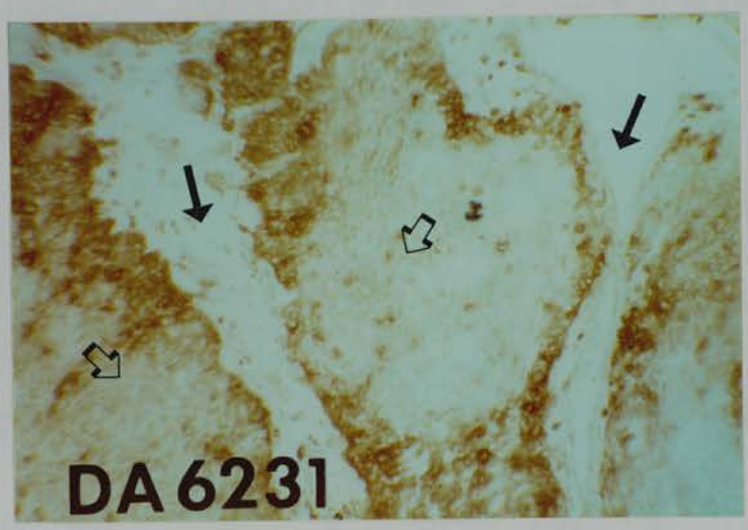

CR 343

Fig.4.3 Enhancement of MHCII staining on what appear to be parenchymal cells. The small cells in the centre of the field are lymphocytes infiltrating vessel walls. High grade astrocytoma, cryostat section, DA6.231 peroxidase, x173

Fig.4.4 Islands of tumour (open arrows) show MHCII staining where they abut upon vascular structures (solid arrows) which are almost entirely unstained. High grade astrocytoma, cryostat section, DA6.231 peroxidase, x270


DA 6231


DA 6231

The pattern of staining of the high grade tumours with each of the seven antibodies and the proportion showing perivascular enhancement is detailed in table 4.1. Every tumour showed some degree of staining with at least one antibody.

Enhanced perivascular staining was a feature in approximately half the tumours for each antibody, but all tumours showed some enhancement with some antibody. For each tumour an analysis was made of the proportion of antibodies which showed an enhanced perivascular reaction. The figures ranged from 14 to 86% with a median value of 57%. The distribution of the results for all 11 tumours is shown in Table 4.2. The highest values were found in the two grade III tumours which showed enhanced perivascular staining with 83% and 86% of antibodies, but two of the three grade IV tumours showed enhancement with 80% of antibodies.

The single low grade astrocytoma showed weak staining with four out of seven antibodies and strong staining with three out of seven. All antibodies showed a pattern of enhanced perivascular staining.

Table 4.1 Pattern of staining for MHC Class II antigens in 11 high grade gliomas.

	Staining			
	0	+	++	Perivascular enhancement
Antibody				
IB5	9.1 (1)	18.2 (2)	54.6 (6)	54.6 (6)
B721	9.1 (1)	45.5 (5)	18.2 (2)	45.5 (5)
CR3/43	0 (0)	9.1 (1)	90.9 (10)	54.6 (6)
DA6147	9.1 (1)	18.2 (2)	63.6 (7)	54.6 (6)
DA6231	9.1 (1)	18.2 (2)	72.7 (8)	54.6 (6)
DA6164	9.1 (1)	36.4 (4)	45.5 (5)	54.6 (6)
Leu10	18.2 (2)	45.5 (5)	36.4 (4)	54.6 (6)

Figures indicate the percentage of tumours showing each degree of staining (actual numbers in brackets). Balance of percentage comprises tumours in which preparations were technically unsatisfactory or unavailable.

Table 4.2 Frequency distribution of perivascular enhancement in 11 high grade gliomas

	% antibodies showing enhancement				
	0-20	21-40	41-60	61-80	81-100
No. of tumours	1	0	5	3	2

INTERIM DISCUSSION

From this series of tumours it was apparent that MHCII expression, often with a perivascular enhancement, was a common phenomenon among glial tumours. Although the frequency of enhancement with the panel of antibodies used appeared to be inversely related to tumour grade the numbers involved were insufficient for this to be stated with any certainty. The differing haplotype specificities of the different antibodies made it clear from the results that MHCII expression is a very heterogeneous phenomenon among glial tumours, and may even differ from area to area in the same tumour as evidenced by cases that showed different degrees of staining with several antibodies and perivascular enhancement with only some. Similar heterogeneity of MHCII expression has been commented upon in B-cell lymphomas (Krajewski et al, 1985; Williamson et al, 1986)

On morphological grounds the bulk of the staining for MHC Class II antigens that was not related to a definable round cell infiltrate was initially interpreted as being on the surface of tumour parenchyma. The MHCII+ round cells had the morphological features of lymphocytes; staining of sections with a B-cell specific antibody (data not shown) had failed to identify any B-cells and so the MHCII+ lymphoid population were considered to be activated T-cells (Ko et al, 1979). Others have subsequently confirmed that B cells are uncommon (Hitchcock et al,

1988) or rare (Rossi et al, 1987) in gliomas.
Against the background of knowledge that glioma-derived cell lines expressed such antigens, and the fact that such antigens could be induced by a range of factors, many derived from lymphocytes the suggestion was made (Franks et al, 1986) that the observed perivascular enhancement might be a reflection of in-vivo induction in response to infiltrating lymphoid cells. A similar association has been postulated between MHCII expression and lymphocytic infiltration in colonic tumours (Lampert et al, 1985). Some support for this view was derived from the observation that tumours showing least perivascular enhancement tended to show less staining for a range of surface lymphocyte antigens (data not shown). An alternative explanation, that the expression was on the surface of macrophages, was thought less likely because of the numbers that would have to be present to account for the intensity of staining. Macrophages had however been described as being present in many gliomas (Morantz et al, 1979; Phillips et al, 1982; Wood et al, 1979) but these studies utilised an in-vitro assay and in the one study in which reference was made to in vivo distribution no pictures were published.

With the finding of such intense MHC class II antigen expression in vivo, short term tissue cultures were thought to be more likely to demonstrate a similar pattern of expression if it genuinely reflected a glial cell

property. Although MHCII expression in colonic carcinoma had been described in a cell line (Lampert et al, 1985) and in cells from fresh explants (Garcia-Espejo et al, 1986) it was recognised that MHC class II expression might prove labile under culture conditions and so a negative finding would not exclude the possibility of glial expression. Accordingly sections from four of the high grade tumours were also examined using a double labelled immunofluorescent technique to determine whether cells expressing MHC class II antigens also expressed the glial intermediate filament GFAP.

RESULTS

In vitro

The 16 day old explant cultures showed a typical outgrowth of process-forming (PF) cells with focal development of abnormal heaps of flattened adherent (FA) cells. Neither GFAP+ PF cells nor FN+ FA cells showed any staining with DA6.231, regardless of their position in relation to the explant. Similarly, in dispersed cell preparations up to 16 days old, no GFAP+ or FN+ cells were seen which also stained with DA6.231.

In the dispersed cell cultures up to 14 days old, and in the 16 day explants, a population of scattered DA6.231+ cells were seen which expressed neither GFAP nor FN. They were generally smaller than glial cell bodies, with a tendency to adhere to GFAP+ cell bodies or processes and

thereby assume an elongated profile (fig.4.5), sometimes with small processes. Where the cells were separate from tissue fragments or other cells they were more likely to assume a rounded shape (fig.4.6).

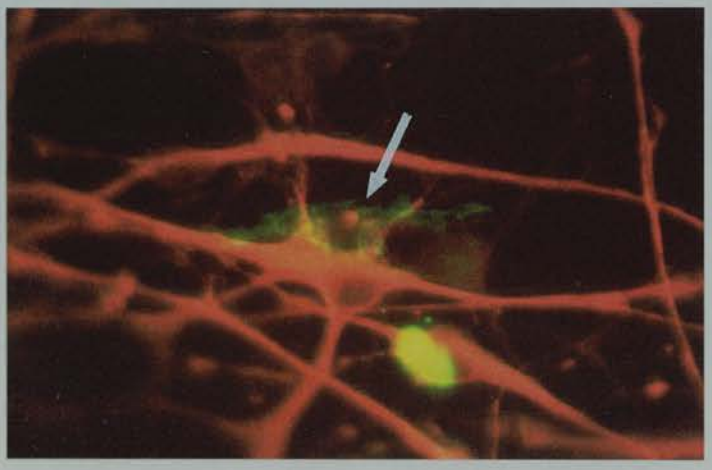
Two cultures from high grade tumours were studied by time lapse photography after 6 days in vitro. Small round highly mobile cells could be seen, distinct from the relatively immobile components of the explant. These cells moved out from and back into the explant, and at the edge of the specimen could be discerned moving in the interstitial spaces between cell processes although the nature of any interaction with glial elements was not clear.

In vivo

Double labelled sections were studied from four of the high grade tumours examined previously. This revealed that, while there was weak expression of MHCII on the surface of GFAP+ parenchymal cells, strong staining was restricted to a population of cells that did not express GFAP+ (fig.4.7), were frequently most prominent around blood vessels and in this location corresponded to cells that also stained strongly either with Dako-macrophage (figs.4.8 & 4.9), or with a marker of mature lymphocytes (T28) (fig.4.10).

Fig.4.5 MHCII+ (green) cell (arrow) adherent to body and process of GFAP+ (orange) glial cell which shows no MHCII staining. The yellow spot in the lower centre of the picture is an artefact. High grade astrocytoma, 16 days in vitro, polyclonal anti-GFAP, monoclonal DA6231, x520

Fig.4.6 MHCII+ cells on substrate base without direct attachment to other cells tend to have rounded profiles. High grade astrocytoma, 16 days in vitro, monoclonal DA6231, x520



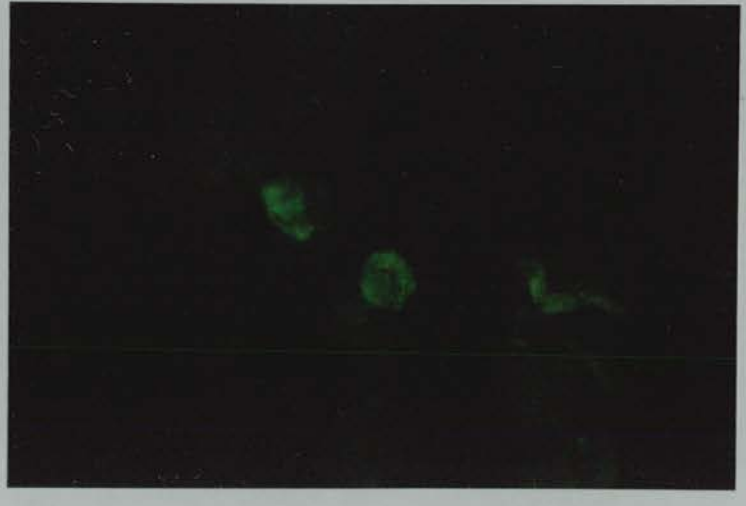
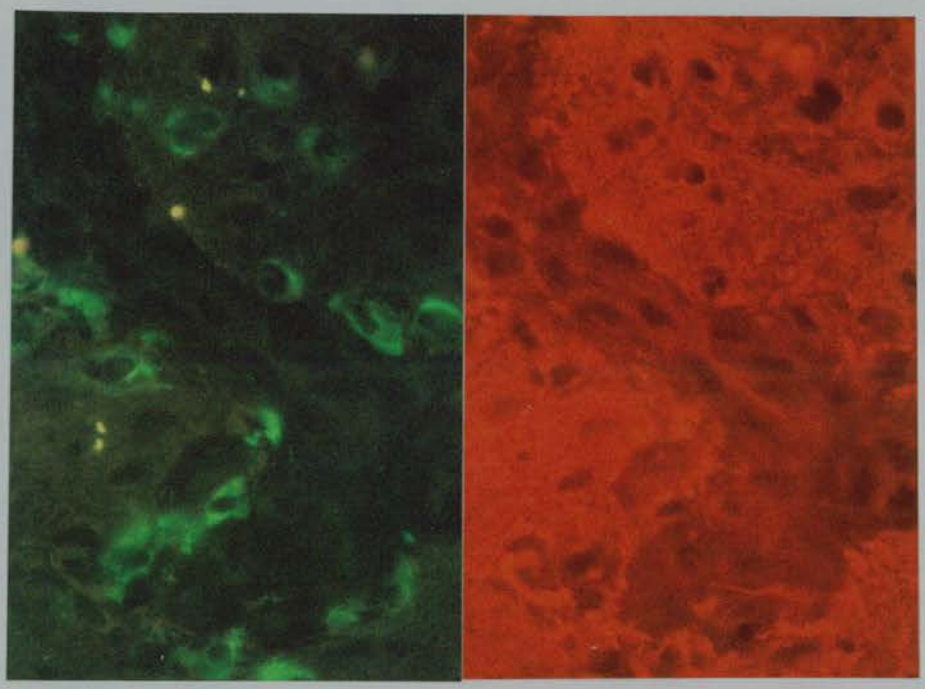


Fig.4.7 MHCII+ cells (left) are relatively process free and do not appear to express GFAP (right) although their enclosure by glial cell processes sometimes makes it difficult to be sure of this. High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal CR3/43, x520

Fig.4.8 Double exposure with macrophage marker (green) and GFAP (orange) clarifies the relationship between the glial and MHCII expressing cells and makes their separation easier. High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal Dako-macrophage, x520



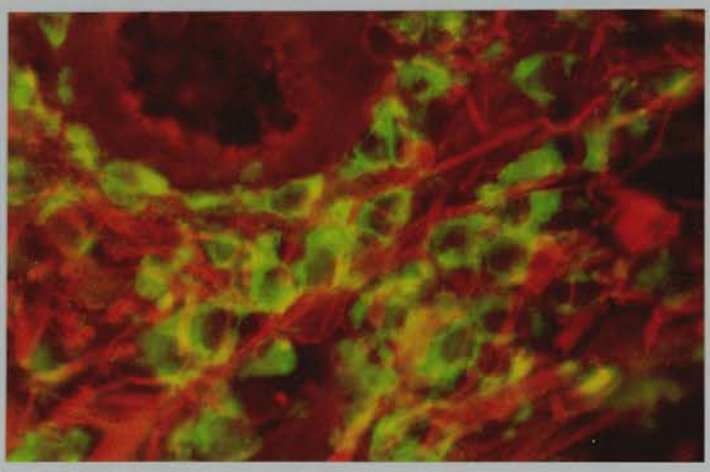
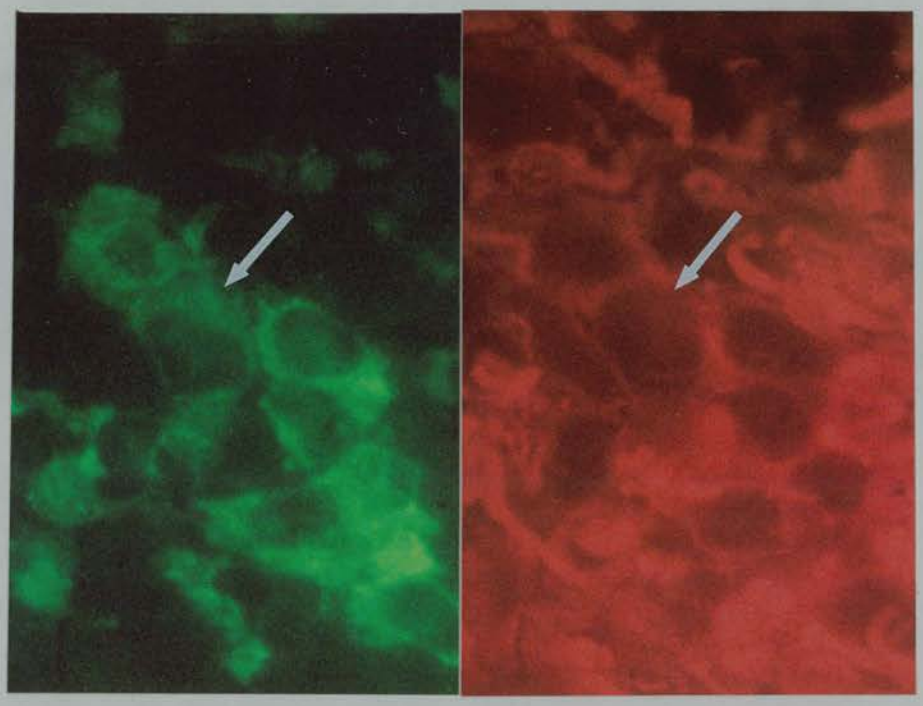
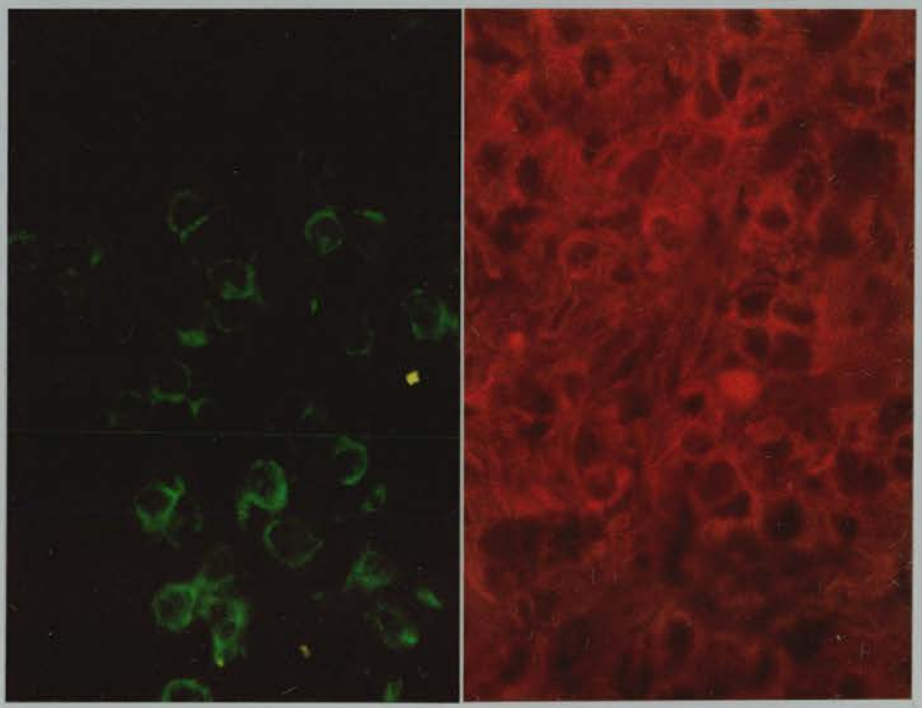


Fig.4.9 At higher magnification the infiltrating cells (green) are seen to be enclosed by GFAP+ (orange) processes, but do not themselves contain GFAP. The same cell is identified in each picture by an arrow. High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal Dako-macrophage, x1040

Fig.4.10 These two pictures from the same field show that a population of the MHCII+ cells also stain with T28 (green), a marker for mature lymphocytes, and are distinct from the GFAP+ (orange) glial cells which enclose them. High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal T28, x520





DISCUSSION

The work presented in this section confirmed the finding of others (Chilosi et al, 1986; Lampson et al, 1986) that MHCII-expression was a common phenomenon in gliomas but raised the question of how much expression was due to glial cells and how much to other elements in the tumour.

At this stage certain conclusions could be drawn. MHCII antigen expression in glial tumours appeared to have at least two components. One was related to the surface of GFAP+ neoplastic glial cells which was diffuse, relatively weak, and appeared to be lost in vitro within 9 days. The second was associated with a cellular infiltrate which consisted of lymphocytes and macrophages. The presence of the latter in any number had not been appreciated because of the difficulties of recognising the cell in routine preparations (Phillips et al, 1982) and of distinguishing it from larger lymphocytes or smaller tumour cells. Such MHCII-expressing cells whose morphology was compatible with their being macrophages could be identified in cultures up to 14 days old.

The in vivo and in vitro expressions of MHCII by glial tumour cells have been compared in an exploration of the susceptibility of such cells to the effect of interferons (Piquet et al, 1986, 1986a). Although MHCII was found in vivo in 9 out of 11 tumours, cells derived in vitro from these same tumours only expressed MHCII in two cases,

suggesting either that expression was lost in vitro, or that the cells being studied represented a different population selected in vitro. Such observations accord with the current studies but the derivation from gliomas of cell lines of confirmed glial nature that also express MHCII (Bigner et al, 1981; Wikstrand et al, 1985), and the direct observation of MHCII- GFAP+ cells in vitro, suggests that culture conditions have an important influence on MHCII expression. The techniques used in this study would be well suited to explore the induction of MHCII by factors known to stimulate such expression in other neoplastic cells (Real et al, 1988).

Whether the expression of MHCII antigens further influences the immune response is of fundamental importance. Interactions between MHCII expressing cells and lymphocytes result in the release of lymphocyte growth factors and it is conceivable that the tumour cells may themselves respond to such factors, a possibility given credence by the observation that in a study of the effect of interferon on cell growth one culture showed a short term enhancement of protein synthesis (Bradley et al, 1983).

Once the contribution of macrophages to the MHCII expression observed in gliomas was appreciated their potential significance in determining tumour growth and the reactions of non-parenchymal elements in vitro was

apparent. A larger study of the relationship between tumour cell proliferation, immune cell infiltrate and MHCII expression was therefore undertaken.

B. RELATIONSHIP OF IMMUNE CELL INFILTRATION, MHC CLASS II ANTIGEN EXPRESSION AND TUMOUR CELL PROLIFERATION IN GLIOMAS.

MATERIALS

MHCII expression (DA6.231), lymphocyte (T11 and T28) and macrophage (Dako-macrophage and Dako-p190,95) counts, and cell proliferation (Ki67) markers were examined in a total of 28 tumours consisting of 17 high grade astrocytomas (15 grade IV and two grade III), five low grade astrocytomas, two meningiomas, one choroid plexus papilloma, one oligodendroglioma, one astroblastoma and one ependymoma. Ki67 staining only was also performed on one metastatic anaplastic carcinoma. Diagnostic classifications were based on serial sections stained H&E from the same tissue as was used in the immunohistochemical analyses.

RESULTS

Qualitative observations

Although some specimens contained areas of infiltrated brain which were analysed for staining pattern, cell counts were, in all cases, carried out in areas of unequivocal primary tumour.

In H&E stained sections the identification of macrophages was not possible (fig.4.11).

The pattern of cellular staining with DA6.231 was similar to that described in the earlier study although resolution was generally better due to increased experience with the

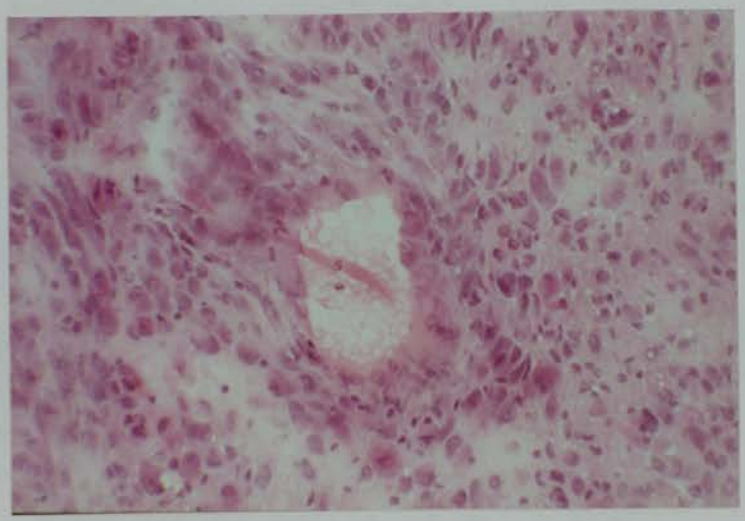
techniques. Two types of cell were discernible, one round in shape with a clear rim of surface staining, and the second larger, sometimes stellate which could have been macrophages or astrocytes. In a number of areas these cells were arranged around blood vessels with the formation of a thin positively stained zone whose distribution (fig.4.12) resembled the astrocytic component of the blood-brain barrier in normal brain.
In addition to this staining of discrete cells was the general tendency of fibrillary or astrocytomatous areas of tumours, and residual neuropil in areas of diffuse infiltration, to stain diffusely positive.

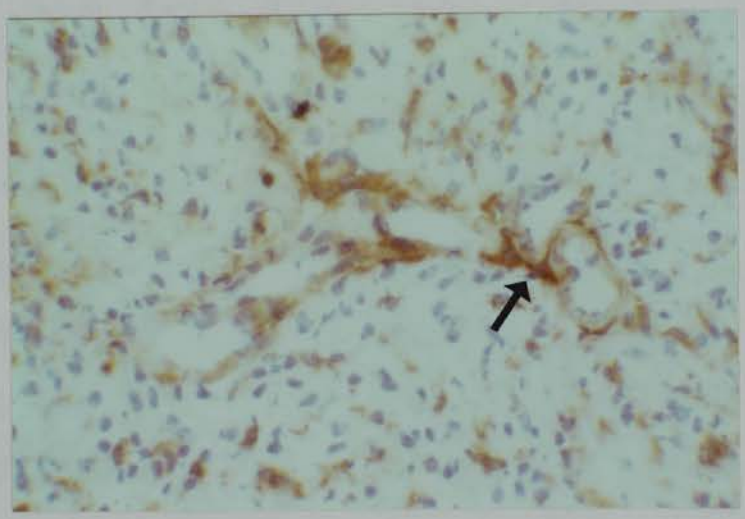
In tumours which showed a diffuse positive staining it was sometimes difficult to distinguish between DA6.231+ cells enclosing unstained cells in their processes and diffuse positivity of most cells (fig.4.13). As a result counts of DA6.231+ cells were not made in five astrocytomas (four grade IV and one low grade) and one meningioma.
The DA6.231+ cells in the ependymoma were situated largely around blood vessels with few in the interstitium, a pattern of positivity reminiscent of the distribution of astrocytes in such tumours.
In the choroid plexus papilloma and both grade III astrocytic tumours there was patchy staining of vascular endothelium with DA6.231. This was not noted in the other tumours and did not appear specifically to be related to local concentrations of other positively stained cells.

Fig.4.11 Although small infiltrating lymphocytes can be identified with some confidence the range of tumour cell size precludes accurate identification of macrophages. High grade astrocytoma, cryostat section, H&E, x173

Fig.4.12 In addition to discrete staining of cells in tumour parenchyma there is strong staining, around small vessels, of cells (arrow) reminiscent of the astrocytes forming the blood-brain barrier of normal brain. High grade astrocytoma, cryostat section, monoclonal DA6.231 peroxidase, x270

Fig.4.13 The diffuse staining of process-bearing cells shown by this tumour makes distinction between stained and unstained elements difficult. High grade astrocytoma, cryostat section, monoclonal DA6.231 peroxidase, x173





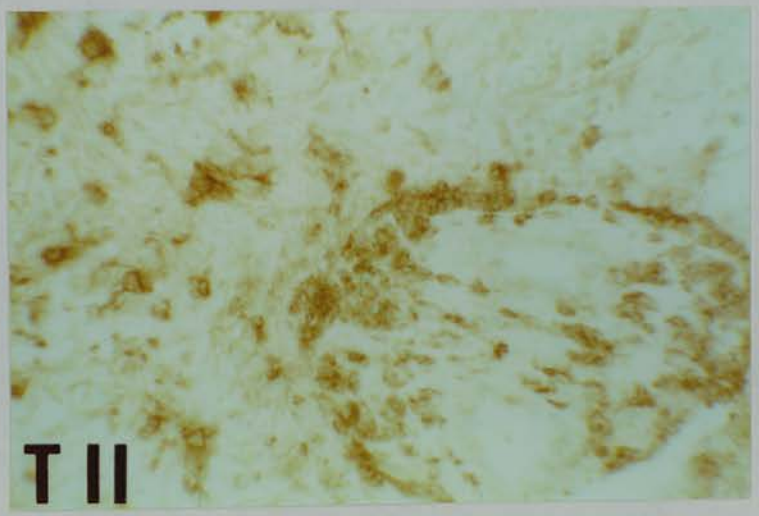
In cases stained with macrophage and lymphocyte markers there was usually sufficient morphological distinction to allow easy delineation of positive and negative cells (fig.4.14). Sometimes perivascular cells stained with the Dako-macrophage antibody (fig.4.15) showed a pattern similar to that of the discrete DA6.231+ cells including the formation of a thin positively stained zone around blood vessels (fig.4.16). In an area of old haemorrhage in the oligodendroglioma foamy macrophages were focally numerous. Although these cells stained with Dako-p150,95 (fig.4.17) those cells in the same area that stained with Dako-macrophage did not have the morphology of lipid-laden cells (fig.4.18).

Ki67 staining was seen to have two types of distribution: one was diffuse throughout the nucleus and the other discrete staining of nucleoli which were frequently multiple (fig.4.19). In some cells dense nucleolar staining was combined with a paler diffuse nuclear reaction. Cells whose nucleus showed any of these patterns were counted as positive. Although vascular structures could be identified in these preparations confident exclusion of all non-glial cells was not felt to be a practical possibility so no attempt was made to distinguish cell types when making counts. However it was noted that positive staining of endothelial cells was very rare and inclusion of proliferating vascular elements was therefore unlikely to give rise to a significant bias.

Fig.4.14 Although there is some variation in the size of cells that are stained with a lymphocyte marker the pattern is essentially one restricted to discrete cells. High grade astrocytoma, cryostat section, monoclonal T11 peroxidase, x173

Fig.4.15 Cells stained for macrophage marker show both a diffuse and a perivascular (arrow) distribution. High grade astrocytoma, cryostat section, monoclonal Dako-macrophage peroxidase, x173

Fig.4.16 Aggregations of cells around small blood vessels (arrows) sometimes resembled the pattern seen with DA6.231 staining astrocytes (see 4.12). High grade astrocytoma, cryostat section, monoclonal Dako-macrophage peroxidase, x270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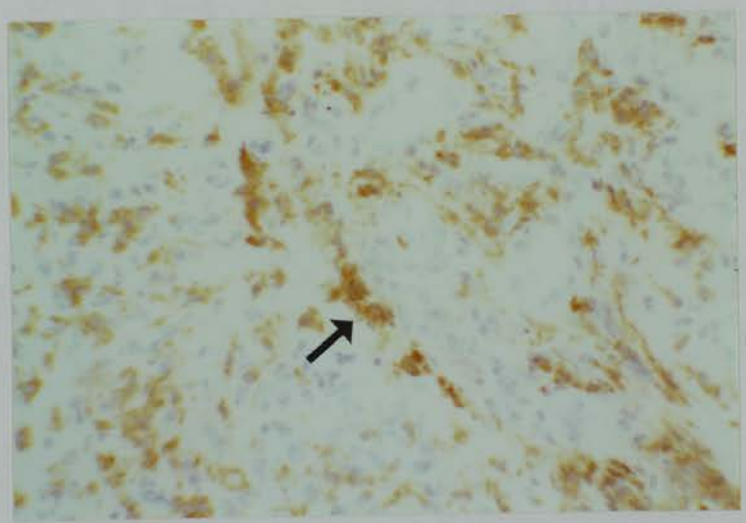


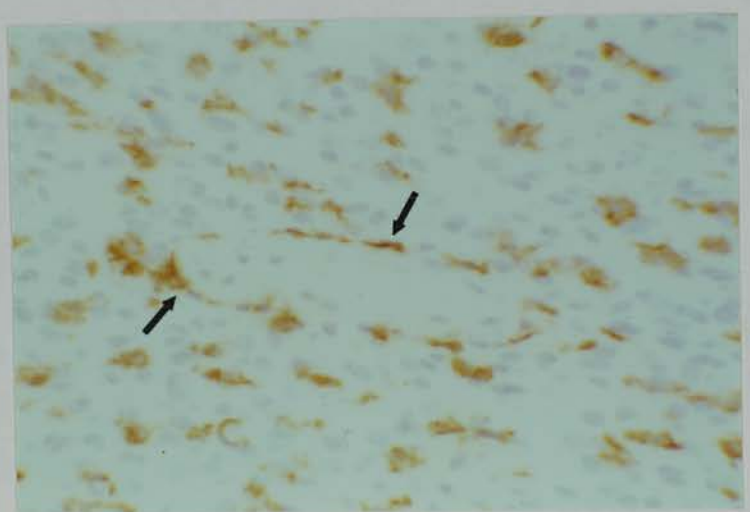
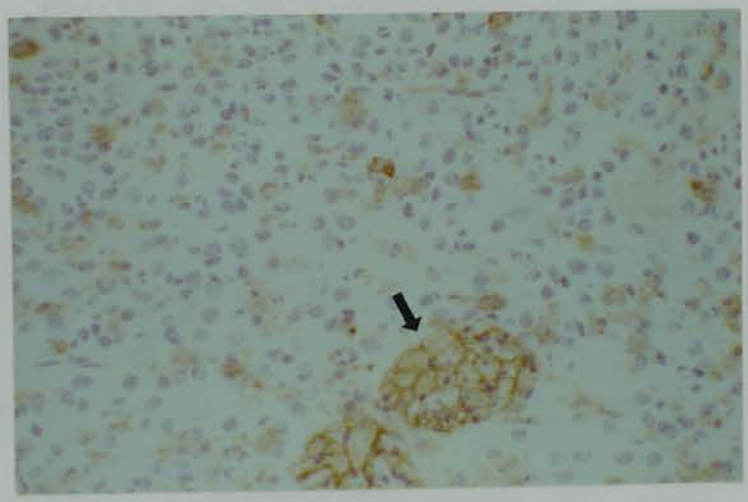
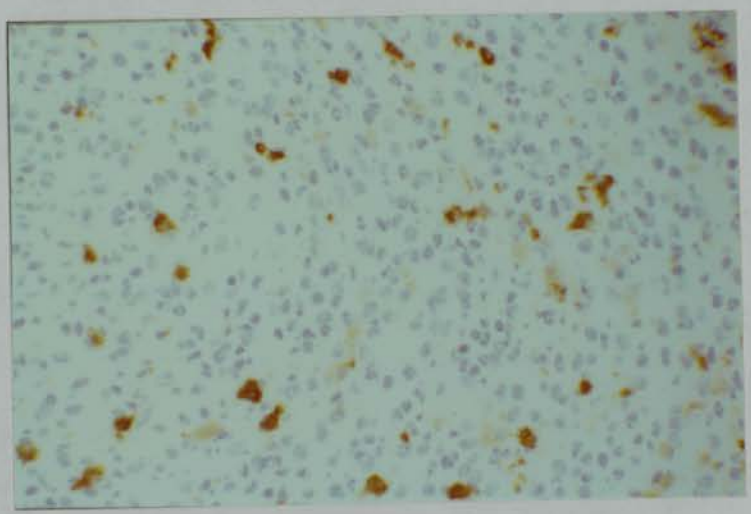


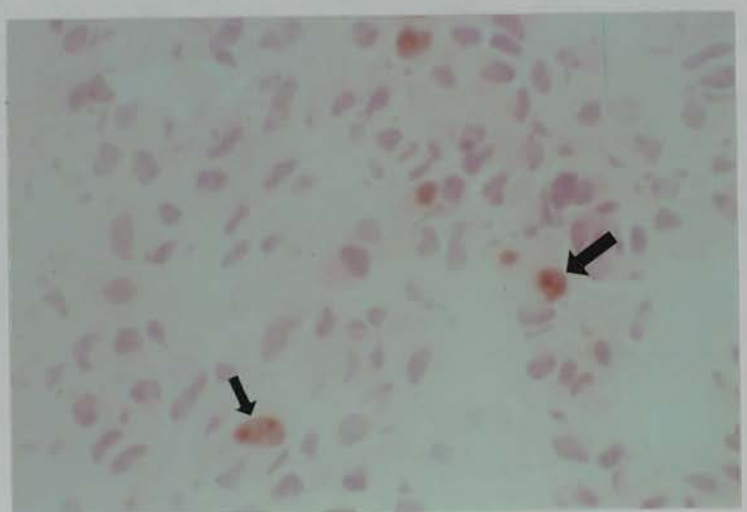
Fig.4.17 Foam cells in an oligodendroglioma (arrow) stain with Dako-p150,95 but staining of other interstitial cells is generally weak. High grade astrocytoma, cryostat section, monoclonal Dako-macrophage peroxidase, x173

Fig.4.18 In a section from the same area as 4.17 there is more staining of interstitial cells with Dako-macrophage, but not of foam cells. High grade astrocytoma, cryostat section, monoclonal Dako-macrophage peroxidase, x173

Fig.4.19 Cells in the proliferative cycle are identified by nuclear (large arrow) and/or nucleolar (small arrow) staining with Ki67. Counterstaining with haematoxylin and eosin allows easy counting of positive and negative cells. High grade astrocytoma, cryostat section, monoclonal Ki67 peroxidase, x432







Fields in which vascular structures formed a dominant component, such as a focus glomeruloid proliferation, were excluded from analysis.

Quantitative observations

The values for the high and low grade astrocytomas for each the six antibodies are detailed in Table 4.3. Although in general the percentages of positively stained cells were lower in the low grade tumours only those for Ki67+ cells were significantly different from the high grade tumours ($p<0.002$; Mann Whitney U test).

Only Ki76 staining was carried out on tissue from the metastasis. Due to inadequate material data were incomplete for a few analyses in the remaining tumours but the available results were used to assess correlation between the percentages of cells staining with the various techniques. The results for tumours other than the astrocytomas are shown in Table 4.4

When the results for all tumours were considered there were significant correlations between the levels of DA6.231+ cells and the levels of both T28+ cells ($r=0.78$, $p<0.001$) and Dako-pl50,95+ cells ($r=0.59$, $p=0.005$).

Table 4.3 Percentage of positively stained cells in high and low grade astrocytomas. The first figure is the median, the second two the range. Difference has been tested for statistical significance tested by Mann Whitney U test.

Antibody	High grade (17 cases)	Low grade (5 cases)	
DA6231	16 5.5 - 42	14 0 - 32.5	NS
T28	2.8 0 - 14.5	1.3 0 - 4.1	NS
T11	1.1 0.3 - 17.3	0.25 0.24 - 1.5	NS
Dako-p150,95	17.9 0.8 - 43	8.7 0 - 26.4	NS
Dako-macrophage	17.7 5.5 - 51.4	15.8 10.9 - 45.6	NS
Ki67	3.8 1 - 18	0.3 0 - 2.1	p<0.002

Table 4.4 Percentage of positively stained cells in seven non-astrocytomas.

	DA6.231	T28	T11	150,95	Dakomp	Ki67
Meningiomas	21.5 *	8.5 2.9	0.1 1.6	* 31.5	19.6 43	0.6 0.3
Choroid plexus papilloma	*	0.6	0.3	3	17	0.3
Oligodendroglioma	9.7	7.8	*	0	4.4	2
Astroblastoma	11.6	0.5	0.3	5.6	12.3	0
Ependymoma	4.9	0.2	*	4.3	3.7	0.9
Metastasis	*	*	*	*	*	3.1

*Data not available

Comparisons were made between the results for Dako-macrophage and Dako-p150,95, and T28 and T11 respectively, to try to assess the degree to which they were each identifying the same populations of macrophage or lymphocytes.

Dako-macrophage tended to identify larger numbers of cells with a mean difference for the two macrophage markers of 8.16% (95% Confidence Limits 11.4, 4.9) which was highly significant ($p<0.0001$, two-tailed t test; 95% Limits of Agreement -9.7, 26); this indicates poor agreement between the two markers and suggests that the two markers do not identify the same population of cells. For the two markers of mature lymphocytes the mean difference was smaller at 1.5% (95% Confidence Limits 2.6, 0.4) but this was still highly significant ($p=0.014$, two-tailed t test; 95% Limits of Agreement of 6.4 and -3.4) again indicating that different populations of cells are being identified.

When the results were restricted to those for the 22 astrocytic lesions the only correlation that persisted was that between the DA6.231+ cells and T28+ cells ($r=0.67$, $p<0.02$). A correlation between the levels of Dako-p150,95+ cells and T28+ cells was found ($r=0.72$, $p<0.01$) that had not been apparent in the larger group. No correlations were found between levels of Ki67+ cells and those stained with any other marker.

C. THE EXPRESSION OF EPITHELIAL GROWTH FACTOR RECEPTOR IN GLIOMAS

EGFR expression was examined in sections from 28 high grade astrocytomas (25 grade IV and three grade III), three low grade astrocytomas and one ganglioglioma. All sections were stained with a double labelled immunofluorescent technique counterstained for GFAP.

30 cultures (nine explants and 21 dispersed cells) from 10 astrocytomas (nine high grade and one low grade) and one ganglioglioma were used to determine expression of EGFR after periods of up to 28 days in vitro. Preparations were double stained with a variety of second antibodies (GFAP, F8RAg, FN) to relate the antigenic profile of cells to EGFR expression.

RESULTS

In vivo

The three low grade astrocytomas and the ganglioglioma showed no staining for EGFR.

Seven of the high grade tumours showed no staining. In the remaining 19 high grade tumours the staining was in one of three recognisable patterns.

i) In five tumours scattered single cells, usually large, process-bearing and GFAP+, showed weak surface staining of

the cell processes and/or the cell body (fig.4.20). These cells were distributed throughout the tumour and did not appear to differ in shape or size from other parenchymal tumour cells.

ii) In four tumours the numbers of cells and their staining intensity were both higher so that small groups of EGFR1+ cells were seen separated by unstained cells (fig.4.21). EGFR1+ cells showed a range of sizes but this did not differ from that shown by EGFR1- cells nor was any difference in GFAP staining apparent between the EGFR1+ and EGFR1- cells.

iii) In 10 tumours staining was uniform and strong corresponding to GFAP+ parenchymal areas (figs.4.22 & 4.23).

In the 28 malignant tumours there was no apparent relation with grade, one grade III tumour being present in each of the three positive groups.

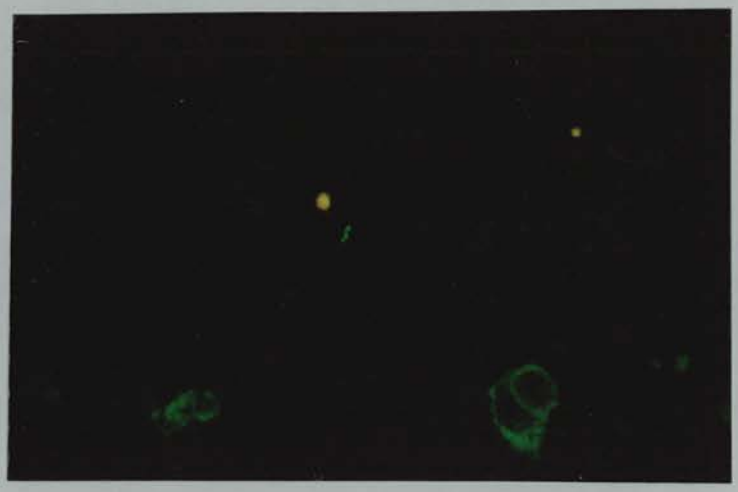
Although the majority of positive staining for EGFR1 was in GFAP+ cells in parenchymal zones two exceptions to this pattern were noted. In three grade IV tumours scattered single GFAP- cells without obvious processes showed EGFR1 staining (fig.4.21). In two grade IV tumours with desmoplasia EGFR1+ cells, which were also GFAP+, were seen in small numbers in the desmoplastic area.

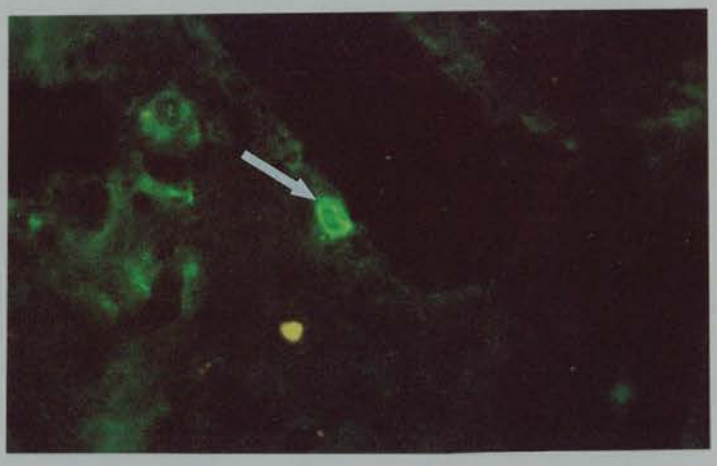
Identifiable vascular structures were unstained in all the 32 tumours examined and in particular no endothelial staining was seen.

Fig.4.20 EGFR expression grade 1. Single EGFR+ cells are surrounded by unstained cells which are in the majority. High grade astrocytoma, cryostat section, monoclonal EGFR1, x520

Fig.4.21 EGFR expression grade 2. EGFR+ cells form small clusters (left) although many cells are still unstained. A single EGFR+ cell (arrow), without processes, lies in close relation to the edge of a small vessel. High grade astrocytoma, cryostat section, monoclonal EGFR1, x520

Fig.4.22 EGFR expression grade 3. The majority of tumour cells stain strongly and uniformly. High grade astrocytoma, cryostat section, monoclonal EGFR1, x520





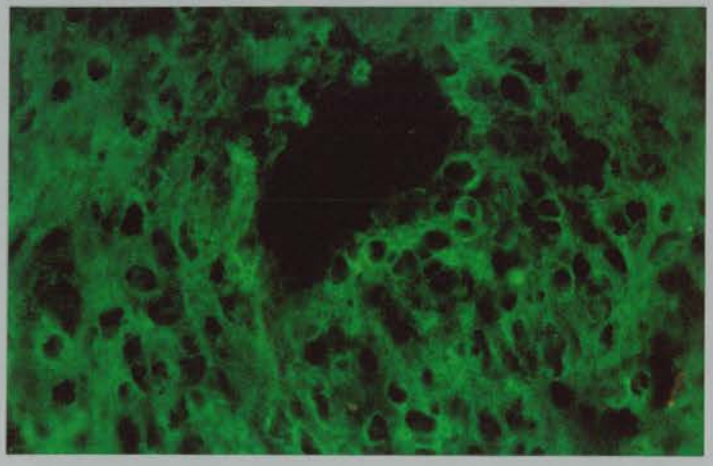
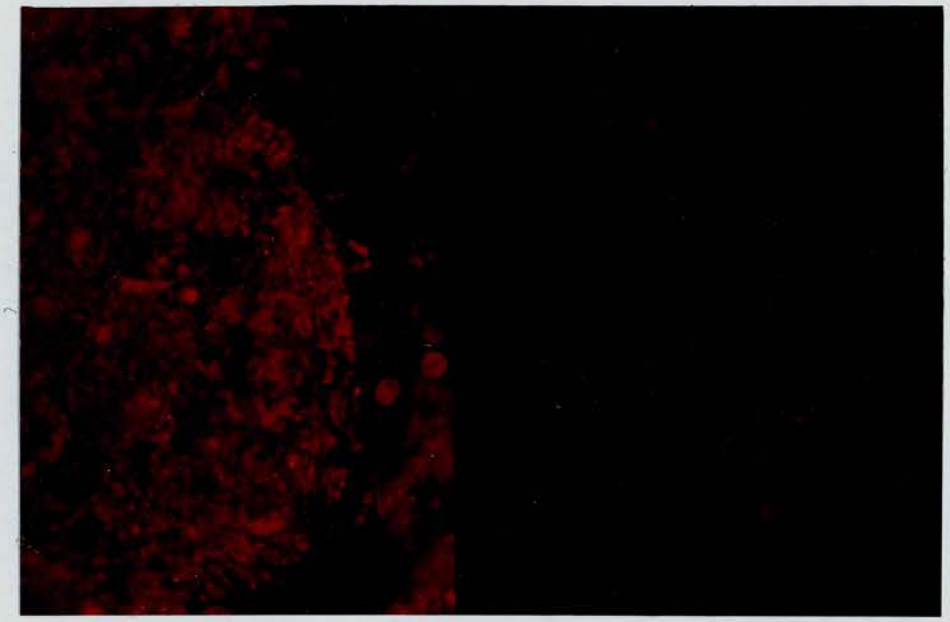


Fig.4.23 The glial nature of the cells expressing EGFR is confirmed by the almost complete correspondence of GFAP expression (orange) and EGFR staining (green). High grade astrocytoma, cryostat section, polyclonal anti-GFAP, monoclonal EGFR1, x330



The differences observed in the distribution of staining grades for EGFR1 between the high and low grade astrocytic tumours were statistically significant ($0.05 > p > 0.02$, Mann Whitney U test).

Relationship with Ki67 staining

In 16 astrocytic tumours (14 high grade and two low grade) data were available to examine the association between EGFR expression and cell proliferation as indicated by Ki67 staining. Ki67 grade was assigned using the 33rd and 66th centiles of the values measured for all gliomas as demarcators of grade. On this basis a low grade (1) comprised values 0-2.7%, medium grade (2) 2.8-4.4% and high grade (3) 4.5-18%. The relationship between EGFR1 grade and Ki67 grade is shown in table 4.5. One-way analysis of variance (Kruskal-Wallis) showed that this distribution did not differ significantly from what would be expected by chance, indicating that no relationship between Ki67 staining and EGFR staining can be identified in the cases examined.

In vitro

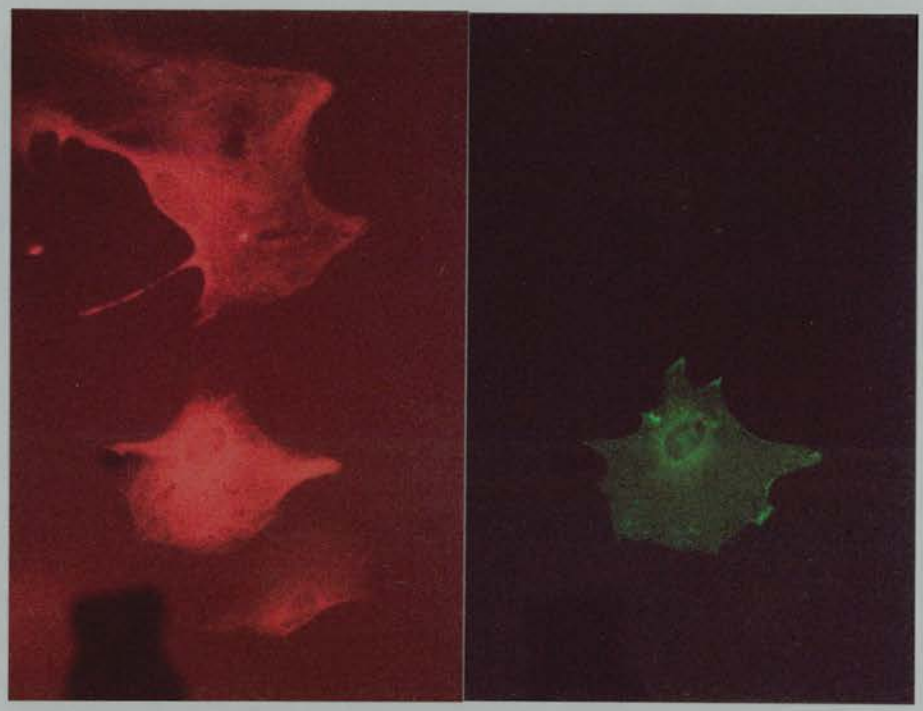
In cultures from nine high grade tumours, ranging in age from 9 to 34 days, neither FN+ nor GFAP+ cells showed any staining with EGFR1 irrespective of whether they were in explant or dispersed cell cultures. Heaps of abnormal FN+ FA cells and typical GFAP+ PF cells were both specifically identified as EGFR1-. In the ganglioglioma GFAP+ cells and

"cobblestone" sheets of F8RAg+ cells were also EGFR1-.
In one culture from a grade III tumour a single GFAP- cell whose morphology was neither process-forming nor typically flattened and adherent showed focal surface staining with EGFR1.
Three cultures from a grade IV tumour contained small numbers of FA cells with uniform surface staining with EGFR1. One culture was examined after 24 days primary culture and the other two 7 days after passage (24 and 28 days total in vitro). In the 24 day cultures the EGFR1+ cells were all GFAP+ (fig.4.24) but in the post-passage preparation the EGFR1 reaction was distinctly focal (fig.4.25). In the older preparation both GFAP+ and GFAP- cells were EGFR+. In neither specimen were typical GFAP+ PF cells stained.

In six high grade tumours correlations were possible between in vivo and in vitro staining. These are shown in Table 4.6.

Fig.4.24 After 24 days in vitro only a subpopulation of GFAP+ cells (left) express EGFR (right). High grade astrocytoma, polyclonal anti-GFAP, monoclonal EGFR1, x520

Fig.4.25 A single GFAP+ cell (left) shows focal EGFR expression (right) on its surface. High grade astrocytoma, 28 days in vitro, first passage, polyclonal anti-GFAP, monoclonal EGFR1, x520



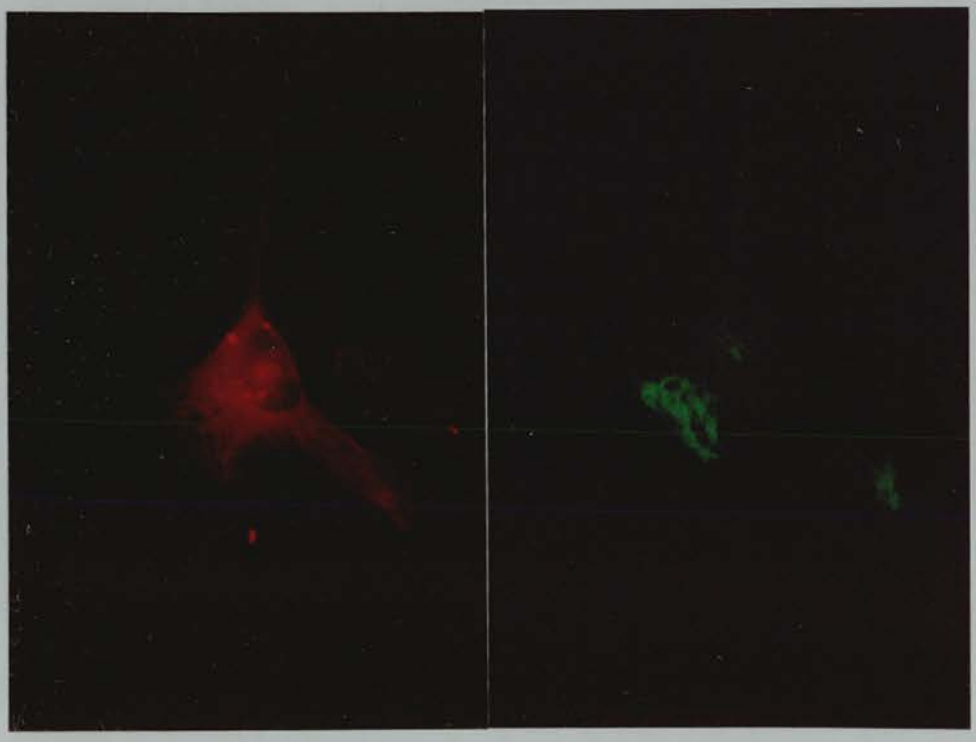


Table 4.5 Relationship between EGFR1 and Ki67 staining grades.

	Ki67		
EGFR1	High	Medium	Low
None	2	-	2*
Low	-	4	-
Medium	-	1	1
High	1	1	4

*This group comprises both the low grade tumours

Kruskal-Wallis one-way analysis of variance showed no significant association between EGFR and Ki67 grades (p=0.5)

Table 4.6 Correlations between in vivo and in vitro expression of EGFR in 6 high grade astrocytomas. Numbers of tumours in each category.

		IN VITRO			
		Uniform Strong	Focal Strong	Focal Weak	Negative
TUMOUR	Uniform Strong		1		1
	Focal Strong				1
	Focal Weak				
	Negative				3

DISCUSSION

The expression of MHC Class II antigens (MHCII) by the cells of tumours from sites other than the brain has been described in lymphomas (Epenetos et al, 1985; Williamson et al, 1986) melanomas (Ruiter et al, 1984; Van Duinen et al, 1986), carcinoma of the breast (Perez et al, 1986; Zuk et al, 1987) and colon (Lampert et al, 1985; Momburg et al, 1986; Moore et al, 1986) and thymomas (Ring et al, 1986). In melanocytes the expression of MHCII has been associated with malignant change and tumour progression, being absent in most naevi but being found with progressively increasing frequency in dysplastic naevi, primary melanomas and metastatic lesions (Ruiter et al, 1984; Van Duinen et al, 1986). Studies of an experimental tumour cell line (Jack et al, 1987) have shown an association of MHCII expression with increased metastatic efficiency and in thymomas there is a suggestion that there is more expression in invasive lesions (Ring et al, 1986). However in breast (Zuk et al, 1987) and colon carcinomas (Momburg et al, 1986) no such association with tumour grade or behaviour has been found.

Although counts of MHCII+ cells were generally higher in high grade tumours difficulties were encountered in localising the expression to the surface of tumour cells rather than of infiltrating macrophages. The marked heterogeneity of expression that was found with a panel of antibodies of differing haplotype specificities, and the

fact that most tumours express some MHCII suggests that there is unlikely to be a simple relationship between MHCII expression and neoplastic progression in glial cells. With the evidence that normal astrocytes have the potential to express MHCII the significance of such expression in tumours is more likely to be related to its influence on any evoked immune reaction than as an indicator of malignant change.

The staining of endothelial cells that was a feature in one choroid plexus papilloma and two grade III astrocytomas has been noted in B cell lymphomas (Epenetos et al, 1985) and in melanomas (Ruiter et al, 1984), and in the latter case this expression contrasted with the lack of expression in adjacent normal vessels. Whether this represents a phenomenon of induction in response to local lymphokines, or an aberrant expression associated with tumour-associated endothelial proliferation is not apparent. The expression of such antigens by endothelial cells would however indicate a potential for MHC-restricted interactions with lymphocytes, the results of which could be the production of factors acting to further stimulate endothelial growth. The rarity of the phenomenon suggests that such expression is not an essential component of tumour angiogenesis in gliomas.

It was apparent from this study that macrophages are present in astrocytic tumours in far larger numbers than

an examination of routinely stained sections would suggest. Similar findings have been recently described by others (Hitchcock et al, 1988; Rossi et al, 1987; Rossi et al, 1988). Although the median values obtained with the two macrophage markers were remarkably similar the wide 95% limits of agreement for the two methods suggest that there are differences in the type of cell identified. The tendency for Dako-macrophage to stain larger numbers of cells in the same tumour is in keeping with the observations of others (Rossi et al, 1987; Rossi et al, 1988) that there were variations in the staining with different markers in some tumours. In vitro studies of macrophage surface antigen expression have shown that the state of activation is reflected in detectable differences in surface antigens (Kreipe et al, 1986). The differential staining observed in some individual tumours, particularly that of the foamy and non-foamy macrophages in the oligodendroglioma, suggests that the antigen identified by each antibody may similarly indicate a different state of activation or function.

Although the mean difference between the values for the two lymphocyte markers was small in absolute terms the 95% limits of agreement were large in comparison with the levels measured for each marker in most tumours. This failure to find agreement between the two markers is perhaps not surprising considering the different Clusters of Differentiation that the two antigens identify (Bernard

et al, 1984); it does however emphasise that the complex heterogeneity of antigen expression in T cells may complicate measures of the degree of lymphocyte infiltration in tumours that utilise immunohistochemical techniques.

The fact that the general levels of positive staining for both lymphocytes and macrophages were higher in high grade than low grade astrocytic lesions is compatible with previously reported observations in this group of lesions using conventionally stained material (Bertrand et al, 1960; Brooks et al, 1978). Hitchcock and Morris (1988) compared high and low grade lesions and likewise found higher percentages of cells staining with macrophage markers and MHCII in high grade lesions, but the differences were not statistically significant. Two semi-quantitative studies of macrophage numbers and MHCII expression in astrocytomas found generally higher levels in the malignant lesions (Rossi et al, 1987) compared to the lower grade tumours (Rossi et al, 1988). Low to moderate numbers of macrophages were present in 38-86% of tumours (depending on the antibody used) although no statistical analyses were made of the differences between grades. In a study of meningiomas a similar increase in macrophage infiltration was noted in atypical lesions when compared to benign tumours (Rossi et al, 1988a). A 12kD factor chemotactic for macrophages has been isolated in vitro from murine and human tumour cells (Botazzi et al,

1983) and greater production of such a substance by more malignant cells could be the basis for a higher level of cellular response in more aggressive tumours. However the complex interactions between lymphocytes infiltrating as part of a primary immune response and macrophages infiltrating in response to lymphokines make it unlikely that a single process is operating. What is of interest however is the fact that there is clear evidence for a qualitatively similar cellular reaction in both low grade and high grade tumours.

It has already been argued that the components of such a reaction may influence the behaviour of non glial elements in vivo and thus in vitro. Macrophage-produced TNF can induce fibroblast proliferation, possibly by activation of similar pathways to EGF although with a different binding site (Vilcek et al, 1986); in vitro activation of macrophages leads to the release of a different factor capable of inducing fibroblasts, smooth muscle cells and endothelium to proliferate when in a prior growth-arrested state (Martin et al, 1981). It is therefore possible that endothelial or other vascular elements in glial tumours of different grades are subject to qualitatively similar stimuli, but differ markedly in their exposure to that part of the angiogenic stimulus that derives from tumour cells. While the latter may be central to the proliferation that characterises malignant gliomas the former could so condition cells that they behave as observed in vitro.

The correlations between the percentages of cells stained with DA6.231 and both T28 and Dako 150,90 suggests that both macrophages and mature lymphocytes contribute to the overall assessment of the number of DA6.231+ cells. Given the same correlation in the glioma subgroup it also could indicate that the lymphocyte response in these tumours is in some way determined by the numbers of MHCII expressing cells, be they macrophages or tumour cells. The first possibility would certainly be in keeping with the sequence of events that follows the development of an immune reaction to foreign antigen; the second is supported by similar observations in breast carcinomas where numbers of T4 subset lymphocytes correlated with the degree of MHCII expression by tumour cells (Zuk et al, 1987).

The correlation between T28+ cells and Dako-150,95+ cells in the gliomas is difficult to explain. The fact that it was not found in the larger group suggests that it reflects the specific circumstances of the immune reaction to gliomas rather than a general association. Certainly it would be in keeping with the suggestion that such a reaction involves lymphocytes and macrophages in a coordinated response to tumour antigen; since T28+ cells include both helper and suppressor T cells clarification would require more detailed analysis of T cell subsets, in large enough numbers of tumours to make allowances for patient age, tumour stage and therapy. The absence of any

correlation between cell proliferation and any of the other cell markers used suggests that there is, in the tumours studied, nothing to support the view that tumour growth is being enhanced by the presence of an immune response.

The ranges of values for Ki67+ cells in the high grade tumours studied correspond well with those reported by Burger et al (1986) who found a range of 1.3 to 12.4% positive cells in high grade lesions. Higher figures of 10-40% with a mean of 28% were reported by Giangspero et al (1987) who considered the lower results to reflect loss of the antigen detected by Ki67 following storage in liquid nitrogen. The material studied by Burger and colleagues had been stored for a maximum of three weeks whereas in the present study storage times occasionally exceeded three years. There was no relationship between length of storage and Ki67 staining and given the correspondence of the present study results with those of Burger et al it would seem that if loss does occur it is not progressive.

The significant difference in cell proliferation that was apparent between high and low grade gliomas extends the observations made by others (Burger et al, 1986; Giangspero et al, 1987), and indicates that a more direct measure of cell proliferation is apparently as efficient as mitotic counts in distinguishing the two groups of

tumours. Such a measure may therefore provide a more sensitive tool in prognostication although this would require prospective testing in a far larger series of lesions. Certainly Burger found that the indices of two fibrillary astrocytomas with nuclear atypia were higher than those of two without this feature.

The relatively low frequency of Ki67 positivity in endothelium that was observed in this study was also noted in Burger's series (Burger et al, 1986) where only a few positive cells were seen in glomeruli of proliferating cells. The conclusion that inclusion of proliferating endothelial cells is unlikely to introduce a significant error is further reinforced by the findings that, despite their appearance, less than 20% of vessels in malignant gliomas contain demonstrably proliferating cells (Nagashima et al, 1987) and have vascular labelling indices that are always much lower than those of tumour cells. These results are however at variance with the study of Davenport et al (1987a) which assessed DNA content of endothelial cells by in-situ photocytometry and found relatively high percentages of cells to be in the cell cycle. These differences may reflect a relative insensitivity of the Ki67 technique, or a low specificity of photocytometry, but do not invalidate the value of Ki67 in making comparisons between tumour types. The proliferative index of the single metastasis in the present series was similar to the median count for the high grade gliomas, as would be expected, and with the

exception of the oligodendroglioma the other tumours all showed low indices in keeping with their known clinical behaviour. The relatively high level for the oligodendroglioma is of considerable interest given the difficulty these lesions present when it comes to predicting behaviour on the basis of morphology (Russell et al, 1977). Studies have shown that the application of conventional markers of malignancy, such as mitotic count, necrosis and vascular proliferation, can be of value in predicting the behaviour of oligodendrogliomas (Burger et al, 1987; Ludwig et al, 1986; Mork et al, 1986; Smith et al, 1983; Wilkinson et al, 1987). The application of Ki67 staining to these tumours may therefore provide information of value.

The finding of EGFR expression in a large proportion of high grade astrocytic tumours confirms the findings of others (Libermann et al, 1984; Wong et al, 1987) but endothelial staining that has been reported to occur in some meningiomas (Shiurba et al, 1988) was not seen in this or other studies (Bigner et al, 1988). The absence of any staining for EGFR in the low grade astrocytomas and the ganglioglioma supports the suggestion (Bigner et al, 1988) that gene amplification is a late event in neoplastic progression.

Although enhanced EGFR gene expression in tumours has been found to be invariably associated with gene amplification

(Libermann et al, 1985, 1985a; Wong et al, 1987), probably largely in the form of double minutes (Bigner et al, 1987), this is a heterogeneous phenomenon at cellular level and tumours which show amplification may still contain up to 15 % of cells in which such amplification is not present (Wong et al, 1987). While EGFR can be demonstrated immunohistochemically in the majority of tumours in which gene amplification has occurred, it can also be found in almost as many tumours in which it has not (Bigner et al, 1988). This may be because the EGFR1 antibody is a more sensitive way to detect increased gene activity when compared to in vitro measures of amplification, or indicate that there is post-translational enhancement of receptor expression that would not be detected by measuring gene copy numbers. Whatever the explanation it indicates that histological methods may not be measuring the same phenomenon as in vitro techniques and any studies of the value of such observations in prognosis must take account of these discrepancies. Comparison of survival times in relation to gene amplification between amplified and non-amplified tumours showed that the former had slightly longer survival times although the differences were not significant (Bigner et al, 1988). With this combination of uncertainty about the significance of gene amplification and the likelihood that EGFR expression in tumour sections is not a reliable measure of gene amplification the failure to demonstrate a clear association between EGFR

expression and proliferative index is not surprising. In vitro studies have suggested that EGFR may directly influence cell motility (Westermark et al, 1982) and so may have a part to play in glial cell infiltration as well as influencing tumour growth.
The few GFAP- cells that stained for EGFR remain unidentified but their morphology and size were more in keeping with their being lymphoid or monocytic rather than neoplastic glial cells.

The numbers of cases in which correlations could be made between in vitro and vivo findings was small, but it is apparent that EGFR expression in vitro is a rare phenomenon irrespective of in vivo expression, and expression in vivo does not guarantee that such expression will be seen in vitro in tissue derived from the same tumour. This may be because gene expression is down regulated under the conditions of culture, although another amplified gene in malignant gliomas, gli, has been shown to persist in vitro (Kinzler et al, 1987) possibly indicating a conferred survival advantage. PA activity has been found to be associated with down regulation of EGFR in squamous carcinoma cells (Gross et al, 1983) and if glial cells were the source of PA in cultures they might be expected to show a reciprocal loss of EGFR. EGF binding has been found to be reduced in both normal and transformed cells when in a growth-arrested state (Robinson et al, 1982) and this has been attributed to

cell cycle dependent expression of the EGF receptor. However the failure in the present study to demonstrate EGFR in the cells that previous studies had shown not to be growth-arrested makes it unlikely that this can be the explanation of the paucity of EGFR expression. The fact that indubitably glial cells in explants never expressed EGFR, even, in one case, when derived from a strongly staining tumour, makes it more likely that initial culture conditions lead to reduced expression in a manner unrelated to cell growth. The finding of GFAP+/EGFR1+ cells in older cultures, albeit in small numbers, does indicate that expression is possible, and given the poor survival of GFAP+ elements in such preparations may indicate some conferred survival advantage although the numbers of cells involved is too small for too much to be made of this observation.

Although cell lines from glioblastomas have been found to have enhanced expression of EGFR in vitro (Blin et al, 1983) this has not been the case with anything like the frequency that would be expected from in-vivo analyses. In one study only 1 out of 22 lines showed enhancement (Filmus et al, 1985) and in another the levels of expression were similar to those found in normal dividing fibroblasts (Steck et al, 1986). These results contrast with the persistence of EGFR expression that has been observed in xenografts (Takahashi et al, 1987) and suggest that EGFR enhancement in gliomas is not as readily

maintained in vitro by gliomas as it is by squamous carcinomas (Filmus et al, 1985) or that there is a selection in vitro of cells that do not have gene amplification, and may not even be glial. In any studies that compare in vitro and in vivo expression of EGFR care must clearly be taken to ensure that any cells expressing, or failing to express, EGFR in vitro are critically identified, and not assumed to be representative of those cells that constitute the majority population in vivo.

Conclusions from studies of immune infiltrates, cell proliferation and growth factor receptor expression.

Although there is clearly a heterogeneity of immune response in astrocytic tumours, with a tendency for this to be more marked in higher grade lesions, the point of most interest in the context of this thesis is that the reactions are qualitatively similar in high and low grade tumours. Cell proliferation indices appear to be an efficient way of discriminating between populations of high and low grade tumours although here is no evidence that the immune response has any direct enhancing, or deleterious, effect on tumour growth.
Significant differences are apparent between the expression of EGFR in high and low grade tumours, although, as with cell proliferation indices, the overlap precludes the use of EGFR staining in the diagnosis of

malignancy in an individual case. The uncertainties surrounding the meaning of immunohistochemically detected EGFR may underly the failure to find any relationship between its expression and cell proliferation despite an association with grade.

It seems likely that vascular elements in high and low grade tumours may be exposed in vivo to factors which, while they might not alter their behaviour in vivo in low grade tumours as grossly as in high grade tumours, may influence their subsequent behaviour in vitro irrespective of tumour type. One other factor that may contribute to their behaviour in vitro is the effect of the carcinogenic agent responsible for the original neoplastic change in the glial cells.

Although some gliomas have been attributed to genetic factors inferred from a familial pattern (Russell et al, 1977), there is a evidence to indicate that in common with much human cancer (Doll et al, 1981) most are probably determined by extrinsic agents. Population studies of astrocytic tumours have shown that migrants from low incidence areas acquire the higher levels of the indigenous population in countries where incidence levels are high (Gold, 1980) and collation studies have suggested that there is a correlation between the incidence levels of brain tumours and levels of atmospheric pollution (Wellington et al, 1979).

Glial tumours can be induced experimentally in animals with viruses (Ogawa et al, 1969; Padgett et al, 1977) or chemicals (Ishida et al, 1975; Seligman et al 1939) but the situation in humans is far from clear. Viral DNA has been identified in some human astrocytic tumours (Ibelgaufts et al, 1982, 1982a), and there is strong epidemiological evidence of an association between industrial vinyl chloride exposure and the development of malignant astrocytoma (Wagoner et al, 1981). Recent work has suggested an association between brain tumour development and exposure to electromagnetic radiation (Thomas et al, 1987); however the groups with high relative risks for astrocytic tumour development were small, had been exposed in an apparently wide range of occupations and no objective data was available on actual, as opposed to presumed, exposure. Nevertheless all astroglial neoplasms occur in parts of the brain where blood-borne agents could gain access and such agents would also, of necessity, have contact with elements of the cerebral vessels.

When one considers the routes of access of environmental carcinogens to the target cell marked contrasts are apparent between the circumstances surrounding many common epithelial tumours and gliomas. In the former the putative agents, be they metabolites in urine in the case of bladder cancer (Berry, 1982), inhaled combustion products of tobacco in the case of the lung (Doll et al 1976), the

bacterial metabolites of bile salts in the case of colon cancer (Hill et al, 1975), or Human Papilloma Viruses in the case of uterine cervix (Brescia et al, 1986), have direct access from the environment without the need to traverse other tissues. This contrasts with the situation that obtains in the brain where any external agent must pass through the blood vessel walls to gain access to glial cells. Only if the active agent is produced locally by enzymatic action after its precursor has gained access would this not be the case.

As a result the carcinogen may effect some damage on the endothelial or other mesenchymal elements that it contacts, even if the damage is insufficient to result in immediate abnormalities of cell behaviour. Such a partial transformation has been observed in the effects of a chemical carcinogen on experimental fibroblasts; cells that appear morphologically to be homogeneously transformed are found to comprise two populations, one fully transformed and tumourigenic, and the other whose abnormal growth pattern can be reversed by ascorbate and which is incapable of tumour formation (Benedict et al, 1982). It is clear therefore that an agent capable of inducing malignant change in cells may effect only partial damage which may be manifest as an abnormality of growth in vitro. Such dormant damage may then only become manifest when a stimulus to proliferate is applied. In the context of astrocytic tumours such manifestations could include an enhanced survival in vitro in the form of cells

whose phenotype was not glial, or an increased liability to malignant progression in vitro or in vivo giving rise to mixed glioblastoma/sarcoma in vivo (Morantz et al, 1976), sarcoma following transplantation of glial tumour (Greene et al, 1968) or tumourigenic cell lines following culture (Rutka et al 1987a). It is of interest that in a study of a virally-induced glial tumour in rats (Groothuis et al, 1980) the incidence of mixed sarcoma/glioma was 7%, which is in the same order as that observed in human series (Morantz et al, 1976); the authors were interested in the possibility that mesenchymal elements might ultimately become transformed as a consequence of their constantly stimulated state and that such a transformation might influence the eventual clinical outcome. In view of the generally poor outlook for patients with malignant gliomas this is unlikely to be a significant consideration unless or until therapy is developed that specifically influences the neoplastic glial elements but leaves the residual non-neoplastic, but nevertheless damaged, mesenchyme intact and liable to further progression.

CHAPTER 5. FINAL DISCUSSION AND CONCLUSIONS

This thesis began with a consideration of the intra-tumoural heterogeneity of neoplastic cells in tumours in general and astrocytic neoplasms in particular. Studies aimed at examining this phenomenon in vitro yielded information on two apparently phenotypically distinct populations of cells whose isolation seemed to depend more on culture methods than on tumour type. Although one population expressed GFAP and was clearly glial the second expressed surface fibronectin (FN), and, although appearing to be transformed on the basis of its growth pattern, had characteristics that raised the possibility of a non-glial origin. A detailed consideration of the arguments for the latter cells being glial or of vascular/mesenchymal origin left the matter unresolved although most of the available evidence was at least consistent with an origin from vascular rather than parenchymal elements in the tumour.

In a search for in vivo correlates of the in vitro phenotypes of the two main cell types no evidence was found to support the view that the FN-expressing cells were derived from a subpopulation of neoplastic cells which were rare in vivo but able to thrive, to the detriment of GFAP-containing cells, under appropriate culture conditions. One phenotypic feature, namely the expression of tissue plasminogen activator (t-PA), was

found, with one exception, to be restricted to vascular elements of astrocytic tumours.

In a further examination of the idea that vascular and neoplastic elements in astroglial tumours are subject to growth stimuli other than those derived from the neoplastic cells themselves, the inter-relations between tumour cell proliferation, immune cell infiltration, growth factor receptor expression and tumour type were studied. The results indicated that although there were quantitative differences between high and low grade tumours with regard to these features most tumours contained an immune cell infiltrate which could be contributing to a stimulus to angiogenesis. Nevertheless the differences in vascular pattern between high and low grade lesions suggest that such a contribution is likely to be overshadowed by the difference in stimuli supplied by high grade malignant cells and the cells of a low grade tumour.

If the FN-expressing cells observed in cultures from both high and low grade astrocytic tumours are derived from mesenchymal rather than glial cells, what are the implications for our understanding of the behaviour of glial tumours in vivo and studies of such lesions in vitro? It is clear that even if these cells are derived from non-neoplastic components they are not entirely normal in their behaviour, or in their genetic makeup

(Kennedy et al, 1987). In serially transplanted human gliomas cells of presumed vascular origin have shown progression to a full neoplastic state (Greene et al, 1968) and may therefore have been damaged by the same agent that induced neoplastic change in the astroglial cells that comprise the parenchymal component of the tumour. How widespread such a phenomenon is in tumours of other sites is not known but, as has been argued earlier, the phenomenon may reflect the route of access of the carcinogen rather than any intrinsic feature of cerebral vessels. Nevertheless the fact that exuberant vascular proliferation and even sarcomatous change has been observed in relation to metastatic tumours (Feigin et al, 1984) does raise the possibility of an intrinsic instability. Of course in the latter circumstance the coincidental prior exposure to an environmental carcinogen, possibly the one that induced the primary extracerebral cancer, with latent damage to endothelium of the brain could not be excluded. In this context it may be significant that vinyl chloride, an agent implicated in the causation of malignant astrocytic tumours (Wagoner et al, 1981), is also responsible for the development of malignant tumours of vascular origin in the form of angiosarcomas of the liver (Creech et al, 1974).

This problem could be further explored by studies that examined the properties and behaviour of vascular elements in tumours of other sites for features similar to those

observed in the studies presented in this thesis. Such work could compare the effect of supernatants from glial tumour cultures (in which glial elements had been shown to predominate) on endothelial cells from non-neoplastic sources and from tissues other than the brain. Of particular interest would be whether the response of normal endothelial cells depended on their tissue of origin. Perhaps in this way the relative importance could be elucidated of prior damage, tumour type and organ of origin in the process of tumour-associated vascular proliferation.

The convenience of tissue culture and its suitability for multiple analyses on a single tumour means that its potential shortcomings must be recognised and account taken of them. The influence of method of culture that was observed in the present work is a clear indication that where cell dispersion techniques are to be used the resulting cultures must be proved to be glial, not simply growing cells that are abnormal phenotypically, or even genotypically. The question of what "gold-standard" marker to use is not an easy one to answer. In general glial markers such as GFAP, high affinity gamma amino butyric acid (GABA) uptake or glutamyl synthetase expression (Freshney et al, 1986) provide proof of a glial nature. In their absence markers of malignancy are used on the assumption that since, in gliomas, only glial cells are neoplastic cells possessing "malignant" properties must be

glial. It would appear however that t-PA expression, loss of contact inhibition, and even angiogenic factor production are not absolute indicators of the neoplastic state, and while GFAP is highly specific when present cells of indubitably glial origin may not express it.

From the work described in this thesis, primary explant culture without repeated passage would seem to provide the greatest chance of glial cells surviving and growing in a mixture reflecting that of the original tumour; studies of DNA content in cultures using such methods (Spaar et al, 1986) have suggested that stable representative populations can be maintained for up to 3 weeks although some cases show overgrowth of diploid cells which are assumed to be of mesenchymal origin. This method however is less reproducible than dispersed cell techniques, is less easy to standardise, and also is less likely to give rise to cells of proven glial nature that can be propagated and repeatedly studied. Techniques such as dispersion and primary culture in conditions that only allow fully independent cells to grow (Shapiro et al, 1985) will provide a greater guarantee that the cells isolated will be glial and neoplastic, but they will inevitably be a highly selected subgroup of the heterogeneous population present in vivo and extrapolation of, for instance, in vitro chemosensitivity tests would require a long period of validity testing. Such methods do however offer the best chance of examining the processes

of neoplastic change and progression in gliomas at a cellular level.

The nature of the interactions between presumed neoplastic glial cells in explant cultures are far from clear; more detailed studies of the ultrastructure of intercellular contacts and their potential to serve a communication function would be rewarding. It would be of interest to know whether isolated FN+ FA cells show other transformed properties such as diminished growth factor requirements and growth in soft agar and whether PA activity from FN+ cells could be more specifically characterised as UK or t-PA.

Even if the FN-expressing cells are derived from non-glial elements they are nonetheless interesting. One implication of the interpretations presented here is that the development of a glial tumour, as opposed to a sarcoma of cerebral mesenchyme, may be a reflection of differential sensitivity of the two tissue elements to an environmental carcinogen, not a absolute difference in response. Furthermore the development of a fully neoplastic state is associated with properties such as angiogenesis-factor production that has implications for the induction of further change in those tissue elements, such as vascular mesenchyme, that have been damaged by the carcinogen but not rendered neoplastic. The third component to consider is the cellular immune reaction to the tumour which may be

induced specifically by abnormal tissue antigens or enhanced by tumour antigens whose function allows tumour/lymphocyte/macrophage interactions which in turn influence both neoplastic and non-neoplastic components of the tumour.

It is clear that human astrocytic tumours are composed not of a homogeneous population of neoplastic cells but are a heterogeneous mixture of interacting glial, mesenchymal and immune cells. Although the end product may be a mass of tissue apparently lacking controls there may be complex interactions and mechanisms determining such growth which, if unravelled, might allow therapeutic control to be imposed. Such tripartite interactions between tumour, stroma, and the immune system probably operate, to varying degrees, in most tumours, and may fruitfully be the subject of further study in human astrocytic neoplasms.

REFERENCES

Adams SL, Sobel ME, Howard BH, Olden K, Yamada K, de Crombrugghe B, Pastan I 1977 Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts. Proceedings of the National Academy of Sciences USA 74:3399-3403

Adams TE, Bodmer G, Bodmer WF 1983 Production and characterisation of monoclonal antibodies recognising the alpha chain subunits of human Ia alloantigens. Immunology 50:613-624

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD 1983 Molecular biology of the cell. Garland Publishing, New York. pp 620-621

Ali SY, Lack CH 1965 Studies on the tissue activator of plasminogen. Distribution of activator and proteolytic activity in the subcellular fractions of rabbit kidney. Biochemical Journal 96:63-74

Ali IU, Hynes RO 1978 Role of disulphide bonds in the attachment and function of the large, external transformation-sensitive glycoprotein at the cell surface. Biochimica et Biophysica Acta 510:140-150

Auerbach R, Sidky YA 1979 Nature of the stimulus leading to lymphocyte-induced angiogenesis. Journal of Immunology 123:751-754

Aulenbacher P, Werling H-O, Paweletz N, Spiess E 1984 Invasive activities of metastasising and non-metastasising tumor cell variants in vitro. Anticancer Research 4:75-82

Azizhan RG, Azizhan JC, Zetter BR, Folkman J 1980 Mast cell heparin stimulates migration of capillary endothelial cells in vitro. Journal of Experimental Medicine 152:931-944

Bailey P, Cushing H 1926 A classification of the glioma group on a histogenetic basis with a correlated study of prognosis. Lippincott, Philadelphia.

Barnard RO, Bradford R, Scott T, Thomas DGT 1986 Gliomyosarcoma. Report of a case of rhabdomyosarcoma arising in a malignant glioma. Acta Neuropathologica 69:23-27

Barrett TB, Gajdusek CM, Schwarz SM, McDougall JK, Benditt EP 1984 Expression of the sis gene by endothelial cells in culture and in vivo. Proceedings of the National Academy of Sciences USA 81:6772-6774

Basta PV, Sherman PA, Ting J P-Y 1987 Identification of an interferon-gamma response region 5' of the human histocompatibility leukocyte antigen DRalpha chain gene which is active in human glioblastoma multiforme cell lines. Journal of Immunology 138:1275-1280

Benedict WF, Wheatley WL, Jones PA 1982 Differences in anchorage dependent growth and tumorigenicities between transformed c3H/10T½ cells with morphologies that are or are not reverted to a normal phenotype by ascorbic acid. Cancer Research 42:1041-1045

BenEzra D 1978 Neovasculogenic ability of prostaglandins, growth factors, and synthetic chemoattractants. American Journal of Ophthalmology 86:455-461

Benke R, Werling HO, Paweletz N 1984 Studies on confrontation of different tumor cells with human diploid fibroblasts. Anticancer Research 4:241-246

Beranek JT, Massayeff R 1986 Hyperplastic capillaries and their possible involvement in the pathogenesis of fibrosis. Histopathology 10:543-551

Bernard A, Boumsell L, Dausset J, Milstein C, Schlossmann SF 1984 Leucocyte typing: report on the First International References Workshop. Berlin. Springer-Verlag. pp143-192

Berry RJ 1982 Carcinoma of the bladder. In Epidemiology of diseases, DL Miller, RDT Farmer (eds), Blackwell, London. pp266-272

Bertrand I, Mannen H 1960 Etude des reactions vasculaire dans les astrocytomes. Revue Neurologique 102:3-19

Bhondeley MK, Mehra RD, Mehra NK, Mohapatra AK, Tandon PN, Roy S, Bijlani V 1988 Imbalances in T cell subpopulations in human gliomas. Journal of Neurosurgery 68:589-593

Bignami A, Dahl D 1985 Glial filaments in brain differentiation and the transient expression of neurofilaments in Purkinje cell development. Annals of the New York Academy of Sciences 455:563-574

Bigner DD, Bigner SH, Ponten J, Westermark B, Mahaley MS, Ruoslahti E, Herschman H, Eng LF, Wikstrand CJ 1981 Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. Journal of Neuropathology and Experimental Neurology 40:201-229

Bigner SH, Burger PC, Wong AJ, Werner MH, Hamilton SR, Muhlbaier LH, Vogelstein B, Bigner DD 1988 Gene amplification in human malignant gliomas: clinical and histopathologic aspects. Journal of Neuropathology and Experimental Neurology 47:191-205

Bigner SH, Friedman HS, Biegel JA, Wikstrand CJ, Mark J, Gebhardt R, Eng LF, Bigner DD 1986 Specific chromosomal abnormalities characterize four established cell lines derived from malignant human gliomas. Acta Neuropathologica 72:86-97

Bigner SH, Wong AJ, Mark J, Muhlbaier LH, Kinzler KW, Vogelstein B, Bigner DD 1987 Relationship between gene amplification and chromosomal deviations in malignant human gliomas. Cancer Genetics and Cytogenetics 29:165-170

Binder B, Spragg J Austen KF 1979 Purification and characterization of human vascular plasminogen activator derived from blood vessel perfusates. Journal of Biological Chemistry 254:1998-2003

Bissel MJ, Barcellos-Hoff MH 1987 The influence of extracellular matrix on gene expression: is structure the message? Journal of Cell Science (Suppl) 8:327-343

Bitterman PB, Rennard SI, Adelberg S, Crystal RG 1983 Role of fibronectin as a growth factor for fibroblasts. Journal of Cell Biology 97:1925-1932

Bjerknes R, Bjerkvig R, Laerum OD 1987 Phagocytic capacity of normal and malignant rat glial cells in culture. Journal of the National Cancer Institute 78:279-288

Bland JM, Altman DG 1986 Statistical method for assessing agreement betwen two methods of clinical measurement. Lancet i:307-310

Blin N, Muller-Brechlin R, Carstens C, Meese E, Zang KD 1983 Enhanced expression of four cellular oncogenes in a human glioblastoma cell line. Cancer Genetics and Cytogenetics 25:285-292

Blom U, Blomgren H, Ullen H, Collins P, von Holst H 1985 Mitogen stimulation of blood lymphocytes from patients with primary intracranial tumors. Correlation with histological tumor type. Anticancer Research 5:343-348

Boethius J, Blomgren H, Collins VP, Greitz T, Strander H 1983 The effect of systemic human interferon-alpha administration to patients with glioblastoma multiforme. Acta Neurochirurgica 68:239-251

Bohling T, Paetau A, Ekblom P, Haltia M 1983 Distribution of endothelial and basement membrane markers in angiogenic tumours of the nervous system. Acta Neuropathologica 62:67-72

Boker D-K 1982 Lymphocytic infiltration in human intracranial tumors - morphological evidence for a host-immune reaction and comparison with the leukocyte migration inhibition (LMI) test. Clinical Neuropathology 1:113-120

Boker D-K, Kalff R, Gullotta F, Weekes-Seifert S, Mohrer U 1984 Mononuclear infiltrates in human intracranial tumors as a prognostic factor: influence of preoperative steroid treatment. Clinical Neuropathology 3:143-147

Borowitz MJ, Weiss MA, Bossen EH, Metzgar RS 1986 Characterization of renal neoplasms with monoclonal antibodies to leukocyte differentiation antigens. Cancer 57:251-256

Botazzi B, Polentarutti N, Balsari A, Boraschi D, Chezzi P, Salmona M, Mantovani A 1983 Chemotactic activity for mononuclear phagocytes of culture supernatants from murine and human tumor cells: evidence for a role in the regulation of the macrophage content of neoplastic tissues. International Journal of Cancer 31:55-63

Bradley NJ, Darling JL, Oktar N, Bloom HJG, Thomas DGT, Davies AJS 1983 The failure of human leucocyte interferon to influence the growth of human glioma cell populations: in vitro and in vivo studies. British Journal of Cancer 48:819-825

Brescia RJ, Jenson AB, Lancaster WD, Kurman RJ 1986 The role of human papillomaviruses in the pathogenesis and histologic classification of precancerous lesions of the cervix. Human Pathology 17:552-559

Brodsky FM 1984 A matrix approach to human class II histocompatibility antigens: reactions of four monoclonal antibodies with the products of nine haplotypes. Immunogenetics 19:179-184

Brooks JJ 1986 Kaposi's sarcoma: a reversible hyperplasia. Lancet ii 1309-1311

Brooks WH, Markesbery WR, Gupta GD, Roszman TL 1978 Relationship of lymphocyte invasion and survival of brain tumor patients. Annals of Neurology 4:219-224

Brown RA, Tomlinson IW, Hill CR, Weiss JB, Phillips P, Kumar S 1983 Relationship of angiogenesis factor in synovial fluid to various joint diseases. Annals of Rheumatic Diseases 42:301-307

Budka H, Majdic O 1985 Shared antigenic determinants between human hemopoietic cells and nervous tissues and tumours. Acta Neuropathologica 67:58-66

Budka H, Majdic O, Knapp W 1985a Cross-reactivity between human hemopoietic cells and brain tumors as defined by monoclonal antibodies. Journal of Neuro-Oncology 3:173-179

Bullard DE, Bigner SH and Bigner DD 1981 The morphologic response of cell lines derived from human gliomas to dibutyryl adenosine 3':5'-cyclic monophosphate. Journal of Neuropathology and Experimental Neurology 40:230-246

Bullard DE, Thomas DGT, Darling JL, Wikstrand CJ, Diengdoh JV, Barnard RO, Bodmer JG, Bigner DD 1985 A preliminary study utilizing viable HLA mismatched cultured glioma cells as adjuvant therapy for patients with malignant gliomas. British Journal of Cancer 51:283-289

Bullard DE, Gillespie GY, Mahaley MS, Bigner DD 1986 Immunobiology of human gliomas. Seminars in Oncology 13:94-109.

Burger PC 1985 The "ideal" classification of pediatric central nervous system neoplasms. Cancer 56:1865-1868

Burger PC 1986 Malignant astrocytic neoplasms: classification, pathologic anatomy and response to treatment. Seminars in Oncology 13:16-26

Burger PC, Schibata T, Kleihues P 1986 The use of the monoclonal antibody Ki-67 in the identification of proliferative cells. Application to surgical neuropathology. American Journal of Surgical Pathology 10:611-617

Burger PC, Green SB 1987 Patient age, histologic features, and length of survival in patients with glioblastoma multiforme. Cancer 59:1617-1625

Burke JM, Ross R 1977 Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. Experimental Cell Research 107:387-395

Cairncross JG 1988 The biology of astrocytoma: lessons learned from chronic myelogenous leukemia. Journal of Neuro-Oncology 5:99-104

Carrel S, de Tribolet N, Gross N 1982 Expression of HLA-DR and common acute lymphoblastic leukaemia antigens on glioma cells. European Journal of Immunology 12:354-357

Carrel S, Schmidt-Kessen A, Giuffre L 1985 Recombinant interferon-gamma can induce the expression of HLA-DR and -DC on DR-negative melanoma cells and enhance the expression of HLA-ABC and tumor-associated antigens. European Journal of Immunology 15:118-123

Chen LB, Summerhayes I, Hsieh P, Gallimore PH 1979 Possible role of fibronectin in malignancy. Journal of Supramolecular Structure 12:139-150

Chilosi M, Gerosa M, Iannucci A, Rosenblum M, Doglioni C, Montagna M, Stevanoni G, Bricolo A, Tridente G 1986 Immunohistochemical demonstration of HLA-DR positive cells in normal and neoplastic brain tissues. I Preliminary report. in Biology of Brain Tumour, Walker MD, Thomas DGT (eds), Martinus Nijhoff, Boston. pp61-66

Chronwall BM, McKeever PE, Kornblith PL 1983 Glial and nonglial neoplasms evaluated on frozen section by double immunofluorescence for fibronectin and glial fibrillary acidic protein. Acta Neuropathologica 59:283-287

Cianciolo GJ, Snyderman R 1986 Effects of tumor growth on host defences. Cancer Metastasis Reviews 5:15-27

Cifone MA, Fidler IJ 1981 Increasing metastatic potential is associated with genetic instability of clones isolated from murine neoplasms. Proceedings of the National Academy of Sciences USA 78:6949-6952

Clark RAF, DellaPelle P, Manseau E, Lanigan JM, Dvorak HF, Colvin RB 1982 Blood vessel fibronectin increases in conjunction with endothelial cell proliferation and capillary ingrowth during wound healing. Journal of Investigative Dermatology 79:269-276

Cohadon F, Aouad N, Rougier A, Vital C, Rival J, Dartigues JC 1985 Histologic and non-histologic factors correlated with survival time in supratentorial astrocytic tumours. Journal of Neuro-Oncology 3:105-111

Cohen S 1962 Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. Journal of Biological Chemistry 237:1555-1562

Collins VP, Brunk U, Fredriksson B.-A, Westermark B 1980 Transmission and scanning electron electron microscopy of whole glioma cells cultured in-vitro. Scanning Electron Microscopy 2:223-230

Colnaghi MI, Canevari S, Menard S 1988 Monoclonal antibodies in immunodiagnosis and immunotherapy of human cancer. European Journal of Surgical Oncology 14:1-7

Cooper MH 1986 Immunogenicity of human malignant gliomas and surface antigen expression. BSc Thesis. Leeds University

Cordingley FT, Bianchi A, Hoffbrand AV, Reittie A, Heslop HE, Vyakarnam A, Turner M, Meager A, Brenner MK 1988 Tumour necrosis factor as an autocrine tumour growth factor for chronic B-cell malignancies. Lancet i 969-971

Coughlin SR, Moskowitz MA, Zetter BR, Antoniades HN, Levine L 1980 Platelet-dependent stimulation of prostacyclin synthesis by platelet-derived growth factor. Nature 288:600-602

Courtoy PJ, Boyle J 1983 Fibronectin in the microvasculature: localization in the pericyte-endothelial interstitium. Journal of Ultrastructural Research 83:258-273

Creech JL, Johnson MN 1974 Angiosarcoma of liver in manufacture of polyvinyl chloride. Journal of Occupational Medicine 16:150-151

Curatolo L, Azzarone B, Fally-Crepin, Morasca L, Maciera-Coelho A 1983 Comparison of fibrin clot retraction with other transformation parameters after hybridization of normal and established cell lines. International Journal of Cancer 31:249-255

Currie GA 1981 Promotion of fibrosarcoma cell growth by proliferation of syngeneic host macrophages. British Journal of Cancer 44:506-513

D'Ardenne AJ, Burns J, Sykes BC, Kirkpatrick 1983 Comparative distribution of fibronectin and type III collagen in normal human tissues. Journal of Pathology 141:55-69

D'Ardenne AJ, Kirkpatrick P, Sykes BC 1984 Distribution of laminin, fibronectin, and interstitial collagen type III in soft tissue tumours. Journal of Clinical Pathology 37:895-904

Dahl D, Bignami A 1982 Immunohistological localization of desmin, the muscle-type 100 A filament protein, in rat astrocytes and Muller glia. Journal of Histochemistry and Cytochemistry 30:207-213

Dahl D, Zapatka S, Bignami A 1986 Heterogeneity of desmin, the muscle-type intermediate filament protein, in blood vessels and astrocytes. Histochemistry 84:145-150

Dano K, Dabelsteen E, Nielsen LS, Kaltoft K, Wilson EL, Zenthen J 1982 Plasminogen activating enzyme in culture glioblastoma cells. Journal of Histochemistry and Cytochemistry 30:1165-1170

Davenport RD, McKeever PE 1987 DNA content and marker expression in human glioma explants. Acta Neuropathologica 74:362-365

Davenport RD, McKeever PE 1987a Ploidy of endothelium in high grade astrocytomas. Analytical and Quantitative Cytology and Histology 9:25-29

De Bault LE 1982 Isolation and characterization of the cells of the cerebral microvessels. in Advances in cellular neurobiology Vol. 3 S Fedoroff & L Hertz (eds). Academic Press, New York. pp339-371

De Larco JE, Todaro GJ 1978 Growth factors from murine sarcoma virus-transformed cells. Proceedings of the National Academy of Sciences USA 75:4001-4005

De Larco JE, Todaro GJ 1980 Sarcoma growth factor (SGF): specific binding to epidermal growth factor (EGF) receptors. Journal of Cellular Physiology 102:267-277

De Murault B, De Tribolet N, Diserens AC, Stavrou D, Mach JP, Carrel S 1985 Phenotyping of 60 cultured human gliomas and 34 other neuroectodermal tumours by means of monoclonal antibodies against glioma, melanoma and HLA-DR Antigens. European Journal of Clinical Oncology 21:207-216

De Ridder LI, Laerum OD, Mork SJ, Bigner DD 1987 Invasiveness of human glioma cell lines in vitro. Relation to tumorigenicity in athymic mice. Acta Neuropathologica 72:207-213

De Tribolet NMF, Hamou J-P, Mach S, Carrel S, Schreyer M 1984 demonstration of HLA-DR antigens in normal human brain. Journal of Neurology Neurosurgery and Psychiatry 47:417-418

Debus E, Weber K, Osborn M 1983 Monoclonal antibodies to desmin, the muscle specific intermediate filament protein. EMBO Journal 2:2305-2312

Deck JHN, Eng LF Bigbee J, Woodcock SM 1978 The role of glial fibrillary acidic protein in the diagnosis of central nervous system tumors. Acta Neuropathologica 42:183-190

Delinassios JG 1983 Uncontrolled growth of tumour stromal fibroblasts in vitro. Experimental Cell Biology 51:201-209

Dewhurst S, Stevenson M, McComb RD, Volsky DJ 1987 Expression of glial fibrillary acidic protein in human glioma cell lines as detected by molecular hybridization. Acta Neuropathologica 73:383-386

Dexter DL, Calabresi P 1982 Intraneoplastic diversity. Biochimica et Biophysica Acta 695:97-112

Doll R, Peto R 1976 Mortality in relation to smoking: 20 years observations on male British doctors. British Medical Journal ii:1525-1536

Doll R, Peto R 1981 The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States. Journal of the National Cancer Institute 66:1191-1308

Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD 1984 Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307:521-527

Duffy PE, Huang Y-Y, Rapport MM, Graf L 1980 Glial fibrillary acidic protein in giant cell tumours of brain and other gliomas. Acta Neuropathologica 52:51-57

Duffy PE, Huang YY, Rapport MM 1982 The relationship of glial fibrillary acidic protein to the shape, motility, and differentiation of human astrocytoma cells. Experimental Cell Research 139:145-157

Durning P, Schor SL, Sellwood RAS 1984 Fibroblasts from patients with breast cancer show abnormal migratory behaviour in vitro. Lancet ii:890-892

Elliot LH, Roszman TL, Brooks WH 1984 Cytokinetic basis for the impaired activation of lymphocytes from patients with primary intracranial tumors, Journal of Immunology 132:1208-1215

Elliot LH, Brooks W, Roszman T 1987 Role of Interleukin-2 (Il-2) and Il-2 receptor expression in the proliferative defect observed in mitogen stimulated lymphocytes from patients with gliomas. Journal of the National Cancer Institute 78:919-922

Elliot LH, Brooks WH, Roszman TL 1987a Activation of immunoregulatory lymphocytes obtained from patients with gliomas. Journal of Neurosurgery 67:231-236

Enblad P, Busch C, Glimelius B, Ponten J, Pahlman L 1985 Antigenic heterogeneity in rectal carcinomas and their secondaries. Anticancer Research 5:610

Eng LF, Rubinstein LJ 1978 Contribution of immunohistochemistry to diagnostic problems of human cerebral tumors. Journal of Histochemistry and Cytochemistry 26:513-522

Eng LF, DeArmond SJ 1982 Immunocytochemical studies of astrocytes in normal development and disease. in Advances in cellular neurobiology Vol. 3, S Fedoroff & L Hertz (eds). Academic Press, New York. pp145-171

Enomoto T, Yamasaki H 1984 Lack of intercellular communication between chemically transformed and surrounding non-transformed Balb/c 3T3 cells. Cancer Research 44:5200-5203

Epenetos AA, Bobrow LG, Adams TE, Collins CM, Isaacson PG, Bodmer WF 1985 A monoclonal antibody that detects HLA-D region antigen in routinely fixed, wax-embedded sections of normal and neoplastic lymphoid tissues. Journal of Clinical Pathology 38:12-17

Erickson CA, Tosney KW, Weston JA, 1980 Analysis of migratory behavior of neural crest and fibroblastic cells in embryonic tissues. Developmental Biology 77:142-156

Evans R 1977 Effect of X-irradiation on host-cell infiltration and growth of a murine fibrosarcoma. British Journal of Cancer 35:557-566

Evans R 1978 Macrophage requirement for growth of a murine fibrosarcoma. British Journal of Cancer 37:1086-1089

Evans R 1984 Regulation of T- and B lymphocyte responses to mitogens by tumor-associated macrophages: the dependency on the stage of tumor growth. Journal of Leukocyte Biology 35:549-559

Evans R, Cullen RT 1984 In situ proliferation of intratumor macrophages. Journal of Leukocyte Biology 35:561-572

Evans R, Duffy T, Cullen RT 1984a Tumor-associated macrophages stimulate the proliferation of murine tumor cells surviving treatment with the oncolytic cyclophosphamide analogue Asta Z-7557: in vivo implications. International Journal of Cancer 34:883-890

Falini B, Pulford K, Erber WN, Posnett DN, Pallesen G, Schwarting R, Annino L, Cafolla A, Canino S, Mori A, Minelli O, Ciani C, Voxes EE, Golomb HM, Delsol G, Stein H, Martelli MF, Grignani F, Mason DY 1986 Use of a panel of monoclonal antibodies for the diagnosis of hairy cell leukaemia. An immunocytochemical study of 36 cases. Histopathology 10:671-687

Feigin I, Allen L, Lipkin L, Gross SW 1958 The endothelial hyperplasia of the cerebral blood vessels with brain tumours, and its sarcomatous transformation. Cancer 11:264-277

Feigin I, Budzilovich GN 1984 Sarcoma arising in metastatic carcinoma in the brain. A second instance. Cancer 54:2047-2050

Fenlon S, Ellis IO, Bell J, Todd JH, Elston CW, Blaney RW 1987 Helix pomatia and Ulex europeus lectin binding in human breast carcinoma. Journal of Pathology 152:169-176

Ffrench-Constant C, Raff MC 1986 Proliferating bipotential glial progenitor cells in adult rat optic nerve. Nature 319:499-502

Fibach E, Landau T, Sachs L 1972 Normal differentiation of myeloid leukemic cells induced by a differentiation-inducing protein. Nature 237:276-278

Filmus J, Pollak MN, Cairncross JG, Buick RN 1985 Amplified, overexpressed and rearranged epidermal growth factor receptor gene in a human astrocytoma cell line. Biochemical and Biophysical Research Communications 131:207-215

Folkman J 1984 What is the role of endothelial cells in angiogenesis? Laboratory Investigation 51:601-604

Folkman J, Merler E, Abernathy C, Williams G 1971 Isolation of a tumor factor responsible for angiogenesis. Journal of Experimental Medicine 133:275-288

Folkman J, Haudenschild C 1980 Angiogenesis in vitro. Nature 288:551-556.

Folkman J, Klagsbrun M 1987 Angiogenic factors. Science 235:442-447

Fontana A, Grob P 1984 Astrocyte-derived interleukin-1-like factors. Lymphokine Research 3:11-16

Fontana A, Hengartner H, de Tribolet N, Weber E 1985 Glioblastoma cells release interleukin 1 and factors inhibiting interleukin 2-mediated effects. Journal of Immunology 132:1837-1844

Forsby N, Collins VP, Westermark B 1985 The spreading of human normal glial and malignant glial cells in culture. Acta Pathologica Microbiologica et Immunologica Scandinavica Sect A 93:235-249

Foulds L 1954 The experimental study of tumor progression: a review Cancer Research 14:327-329

Frame MC, Freshney RI, Vaughan PFT, Graham DI, Shaw R 1984 Interrelationship between differentiation and malignancy-associated properties in glioma. British Journal of Cancer 49:269-280

Frank E, Pulver M, De Tribolet N 1986 Expression of Class II major histocompatibility antigens on reactive astrocytes and endothelial cells within the gliosis surrounding metastases and abscesses. Journal of Neuroimmunology 12:29-36

Franks AJ 1988 Diagnostic manual of central nervous system tumours. Churchill Livingstone, Edinburgh. pg 34

Franks AJ, Burrow HM 1986 In vitro heterogeneity in human gliomas. Are all transformed cells of glial origin? Anticancer Research 6:625-630

Franks AJ, Bird CC 1986a Expression of MHC class II and lymphocyte antigens by malignant human gliomas. Neuropathology and Applied Neurobiology 12:615

Franks AJ, Ellis E 1989 Immunohistochemical localisation of tissue plasminogen activator in human CNS tumours. British Journal of Cancer, in press

Franks LM 1983 The neoplastic cell and its analysis by cell hybridization. In Gene expression in normal and transformed cells. JE Celis and R Bravo (eds). Plenum Press, New York. pp141-176

Franks LM, Carbonell AW, Hemmings VJ, Riddle PN 1975 Metastasising tumors from serum-supplemented and serum-free cell lines from a C57B mouse lung tumor. Cancer Research 36:1049-1055

Frater-Schroder M, Risau W, Hallman R, Gautschi P 1987 Tumor necrosis factor type alpha, a potent inhibitor of endothelial cells growth in vitro, is angiogenic in vivo. Proceedings of the National Academy of Sciences USA 84:5277-5281

Frei K, Bodmer S, Schwerdel C, Fontana A 1985 Astrocytes of the brain synthesise interleukin 3-like factors. Journal of Immunology 135:4044-4047

Frei K, Bodmer S, Schwerdel C, Fontana A 1986 Astrocyte-derived interleukin 3 as a growth factor for microglia cells and peritoneal macrophages. Journal of Immunology 137:3521-3527

Freshney RI 1980 Tissue culture of glioma of the brain. Ch.3 in Brain tumours. Scientific basis, clinical,investigation and current therapy. Butterworth, London pp21-50.

Freshney RI 1985 Induction of differentiation in neoplastic cells. Anticancer Research 5:111-130

Freshney RI, Frame MC, MacDonald CM, Hart E, Shaw R, Hassanzadah M, Freshney MG 1985 Markers of the malignant glial phenotype. Progress in Experimental Tumour Research 29:12-16

Freshney RI, Frame MC, Vaughan PFT, Graham DI 1986 Phenotypic modification of human glioma. Ch. 5 in Biology of Brain Tumour, MD Walker and DGT Thomas (eds). Martinus Nijhoff, Boston

Friedlander ML, Hedley DW, Taylor IW 1984 Clinical and biological significance of aneuploidy in human tumours. Journal of Clinical Pathology 37:961-974

Fromer CH, Klintworth GK, 1975 An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. I comparison of experimental models of corneal vascularization. American Journal of Pathology 79:537-554

Fromme HG, Voss B, Pfautsch M, Grote M, von Figura K, Beeck H 1982 Immunoelectron-microscopic study on the location of fibronectin in human fibroblast cultures. Journal of Ultrastructure Research 80:264-269

Fulling KH, Garcia DM 1985 Anaplastic astrocytoma of the adult cerebrum. Prognostic value of histologic features. Cancer 55:928-931

Furcht LT, Mosher DF, Wendelschafer-Crabb G, Woodbridge PA, Foidart JM 1979 Dexamethasone-induced accumulation of a fibronectin and collagen extracellular matrix in transformed human cells. Nature 277:393-395

Furshplan J, Potter DD 1968 Low resistance junctions between cells in embryos and tissue culture. Current Topics in Developmental Biology 3:95-127

Gahmberg CG, Kiehn D, Hakomori S 1974 Changes in a surface-labelled galactoprotein and in glycolipid concentrations in cells transformed by a temperature-sensitive polyoma virus mutant. Nature 248:413-415.

Gajdusek CM, Schwartz SM 1982 Ability of endothelial cells to condition culture medium. Journal of Cellular Physiology 110:35-42

Garcia-Espejo R, Alonso MC, Solana R et al 1986 Expression of HLA molecules on cells from fresh explants of human digestive tract cancer. Journal of Immunogenetics 13:211-217

Gately MK, Glaser M, McCarron RM, Dick SJ, Dick MD, Mettetal RW, Kornblith PL 1982 Mechanisms by which human gliomas may escape cellular immune attack. Acta Neurochirurgica 64:175-197

Gazso L, Afra D, Muller W 1978 Supratentorial recurrences of gliomas. Tissue culture studies with astrocytomas and oligodendrogliomas. Acta Neuropathologica 44:135-139

Geder L, Kreider JW, Towfighi J, Page RB, Ladda RL, Brennan RW, Rapp F 1987 Effect of differentiation inducers on growth characteristics of human glioma cell lines. Journal of Neuro-Oncology 5:251-271

Gerdes J, Schwab U, Lemke H, Stein H 1983 Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with proliferation. International Journal of Cancer 31:13-20

Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H 1984 Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki67. Journal of Immunology 133:1710-1715

Ghandour S, Langley K, Gombos G, Hirn M, Hirsch MR, Goridis C 1982 A surface marker for murine vascular endothelial cells defined by monoclonal antibody. Journal of Histochemistry and Cytochemistry 30:165-170

Giangaspero F, Doglioni C, Rivano MT, Pileri S, Gerdes J, Stein H 1987 Growth fraction in human brain tumors defined by the monoclonal antibody Ki-67. Acta Neuropathologica 74:179-182

Gilles FH 1985 Classifications of childhood brain tumors. Cancer 56:1850-1857

Gimbrone MA, Leapman SB, Cotran RS, Folkman J 1972 Tumor dormancy in vivo by prevention of neovascularization. Journal of Experimental Medicine 136:261-276

Giulian D, Lachman LB 1985 Interleukin-1 stimulation of astroglial proliferation after brain injury. Science 228:497-498

Gold EB 1980 Epidemiology of brain tumors. In Reviews of cancer epidemiology, Vol 1, EM Lilienfield (ed), Elsevier. pp246-259

Goodall CM, Sanders AG, Shubik P 1965 Studies of vascular patterns in living tumors with a transparent chamber inserted in hamster cheek pouch. Journal of the National Cancer Institute 35:497-521

Gospodarowicz D, Greenburg G, Birdwell CR 1978 determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Research 38:4155-4171

Gospodarowicz D, Ill C 1980 Extracellular matrix and control of proliferation of vascular endothelial cells. Journal of Clinical Investigation 65:1351-1364

Gospodarowicz D, Neufeld G, Schweigerer L 1986 Molecular and biological characterisation of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Differentiation 19:1-17

Greene HSN, Harvey EK 1968 Development of sarcomas from transplants of the hyperplastic stromal endothelium of glioblastoma multiforme. American Journal of Pathology 53:483-499

Greiner JW 1986 Modulation of antigen expression in human tumor cell populations. Cancer Investigation 4:239-256

Greiner JW, Tobi M, Fisher P Langer JA, Pestka S 1985 Differential responsiveness of cloned mammary carcinoma cell populations to human recombinant leucocyte (alpha) interferon mediated enhancement of tumor antigen expression. International Journal of Cancer 36:159-166

Grinnel F, Feld MK 1980 Spreading of human fibroblasts in serum-free medium: inhibition by dithiotheitol and the effect of cold insoluble globulin (fibronectin). Journal of Cellular Physiology 104:321-334

Groothuis DR, Fischer JM, Vick NA, Bigner DD 1980 Experimental gliomas: an autoradiographic study of the endothelial component. Neurology 30:297-301

Gross JL, Krupp MN, Rifkin DB, Lane MD 1983 Down-regulation of epidermal growth factor receptor correlates with plasminogen activator activity in human A431 epidermoid carcinoma cells. Proceedings of the National Academy of Sciences USA 80:2276-2280

Gross JL, Moscatelli DA, Rifkin DB 1983a Increased capillary endothelial cell protease activity in response to angiogenic stimuli in-vitro. Proceedings of the National Academy of Sciences USA 80:2623-2627

Gross JL, Behrens DL, Mullins DE, Kornblith PL, Dexter DL, 1988 Plasminogen activator activity in human glioma cells and modulation by sodium butyrate. Cancer Research 48:291-296

Guerry D, Alexander MA, Herlyn MF, Zehngebot LM, Mitchell KF, Zmijewski CM, Lusk EJ 1984 HLA-DR histocompatibility leukocyte antigens permit cultured human melanoma cells from early but not advanced disease to stimulate autologous lymphocytes. Journal of Clinical Investigation 73:267-271

Gullino PM 1986 Considerations of the angiogenic response. Anticancer Research 6:153-158

Guner M, Freshney RI, Morgan D, Freshney MG, Thomas DGT, Graham DI 1977 Effects of dexamethasone and betamethasone on in-vitro cultures from human astrocytomas. British Journal of Cancer 35:439-447

Guy K, van Heyningen V, Cohen BB, Deane DL, Steel CM 1982 Differential expression and serologically distinct subpopulations of human Ia like antigens detected with monoclonal antibodies to Ia alpha and beta chains. European Journal of Immunology 12:942-948

Haaugen A, Laerum OD, Bock E 1981 Responsiveness of fetal rat brain cells in culture: an ultrastructural study. Brain Research 150:225-238

Haggie JA, Sellwood RA, Howell A, Birch JM, Schor SL 1987 Fibroblasts from relatives of patients with hereditary breast cancer show fetal-like behaviour in vitro. Lancet i:1455-1457

Harris-Hooker SA, Gajdusek CM, Wight TN, Schwartz SM 1983 Neovascular responses induced by cultured aortic endothelial cells. Journal of Cellular Physiology 114:302-310

Hayek A, Culer FL, Beattie GM, Lopez AD, Cuevas P, Baird A 1987 An in-vivo model for the study of the angiogenic effects of Basic Fibroblast Growth Factor. Biochemical and Biophysical Research Communications 147:876-880

Hayman EG, Engvall E, Ruoslahti E 1980 Butyrate restores fibronectin at cell surface of transformed cells. Experimental Cell Research 127:478-481

Haynes LW, Davis BE, Mitchell J, Weller RO 1978 Cell proliferation in explant cultures of malignant gliomas. Acta Neuropathologica 42:87-90

Hedman K, Vaheri A, Wartovaara J 1978 External fibronectin of cultured human fibroblasts is predominantly a matrix protein. Journal of Cell Biology 76:748-760

Heldin C-H, Westermark B, Wateson A 1981 Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proceedings of the National Academy of Sciences USA 78:3664-3668

Heppner GH, Miller BE, Miller FR 1983 Tumor subpopulation interactions in neoplasms. Biochimica et Biophysica Acta 695:215-226

Herpers MJHM, Budka H, McCormick D 1984 Production of glial fibrillary acidic protein (GFAP) by neoplastic cells: adaptation to the microenvironment. Acta Neuropathologica 64:333-338

Herpers MJHM, Raemaekers FCS, Aldeweireldt, Moesker O, Sloof J 1986 Co-expression of glial fibrillary acidic protein- and vimentin-type intermediate filaments in human astrocytomas. Acta Neuropathologica 70:333-339

Hill MJ, Drasar BS, Williams REO, Meade TW, Cox AG, Simpson JEP, Morson BC 1975 Faecal bile acids and clostridia in patients with cancer of the large bowel. Lancet i:535-538

Hince TA, Roscoe JP 1980 Differences in pattern and level off plasminogen activator production between a cloned cell line from an ethylnitrosourea-induced glioma and one from normal adult rat brain. Journal of Cellular Physiology 104:199-207

Hirano A, Zimmerman HM 1972 Fenestrated blood vessels in a metastatic renal carcinoma in the brain. Laboratory Investigation 26:465-468

Hirayama M, Yokochi T, Shimokata K, Iida M, Fujiki N 1986 Induction of human leucocyte antigen-A,B,C and -DR on cultured human oligodendrocytes and astrocytes by human gamma-interferon. Neuroscience Letters 72:369-374

Hirschberg H 1984 Endothelial growth factor production in cultures of human glioma cells. Neuropathology and Applied Neurobiology 10:33-42

Hitchcock ER, Morris C 1988 Mononuclear cell infiltration in central portions of human astrocytomas. Journal of Neurosurgery 68:432-437

Hockel M, Sasse J, Wissler JH 1987 Purified monocyte-derived angiogenic substance (angiotropin) stimulates migration, phenotypic changes, and "tube formation" but not proliferation of capillary endothelial cells in-vitro. Journal of Cellular Physiology 133:1-13

Holthofer H, Virtanen I, Kariniemi A-L, Hormia M, Lindner E, Miettinen A 1982 Ulex europaeus I lectin as a marker for vascular endothelium in human tissues. Laboratory Investigation 47:60-66

Horan Hand P, Colcher D, Salomon D, Ridge G, Noguchi P, Schlom J 1985 Spatial configuration of carcinoma cell populations influences the expression of a tumor associated glycoprotein. Cancer Research 45:833-840

Hoshino T, Nomura K, Wilson CB, Knebel KD, Gray JW 1978 The distribution of nuclear DNA from human brain-tumor cells. Journal of Neurosurgery 49:13-21.

Hynes RO 1980 Fibronectin and its relation to cellular structure and behaviour. ch.10 in Cell Biology of the Extracellular Matrix, ED Hay (ed). Plenum Press, London. pp 295-334

Hynes RO, Byer JM 1974 Density and cell cycle dependence of cell surface proteins in hamster fibroblasts. Cell 3:113-120

Hynes RO, Destree AT 1978 Relationship between fibronectin (LETS protein) and actin. Cell 15:875-886

Ibelgaufts H, Jones KW, Maitland N, Shaw JF 1982 Adenovirus-related RNA sequences in human neurogenic tumours. Acta Neuropathologica 56:113-117

Ibelgaufts H, Jones KW 1982a Papovavirus-related RNA sequences in human neurogenic tumours. Acta Neuropathologica 56:118-122

Ingram M, Shelden CH, Jaques S, Skillen RG, Bradley WG, Techy GB, Freshwater DB, Abts RM, Rand RW 1987 Preliminary clinical trial of immunotherapy for malignant glioma. Journal of Biological Response Modifiers 6:489-498

Ishida Y, Tamura M, Kanda H, Okamoto K 1975 Histopathological studies of the nervous system tumors in rats induced by N-nitroso-methyl-urea. Acta Pathologica Japan 25:385-401

Jack AS, McVeigh KL 1987 A demonstration of a strain related restriction effect in the formation of experimental metastases. Journal of Pathology 152:37-45

Jacobs SK, Wilson DJ, Kornblith PL, Grimm EA 1986 In vitro killing of human glioblastoma by interleukin-2-activated autologous lymphocytes. Journal of Neurosurgery 64:114-117

Jacobsen PF, Jenkyn DJ, Papadimitriou JM 1987 Four permanent cell lines established from human malignant gliomas: three exhibiting striated muscle differentiation. Journal of Neuropathology and Experimental Neurology 46:431-450

Jiwa M, Van den Hoof A 1985 The possible role of stromal influences in tumour cell heterogeneity. histology and fibronectin pattern of the Yoshida sarcoma of the rat. Anticancer Research 5:273-276

Jones TR, Ruoslahti E, Schold SC, Eng LF, Bigner DD 1980 Fibronectin (FN) and glial fibrillary acidic protein (GFAP) expression in human anaplastic gliomas (HGl) grown in athymic "nude" mice. Journal of Neuropathology and Experimental Neurology 39:365

Jones TR, Bigner SH, Schold SC, Eng LF, Bigner DD 1981 Anaplastic human gliomas grown in athymic mice. American Journal of Pathology 105:316-327

Jones TR, Ruoslahti E, Schold SC, Bigner DD 1982 Fibronectin and glial fibrillary acidic protein expression in normal human brain and anaplastic human gliomas. Cancer Research 42:168-177

Kasai T, Pollak OJ 1964 Smooth muscle cells in aortic cultures of untreated and cholesterol-fed rabbits. Zeitscrift fur Zelforschung und Mikroskopische Anatomie 62:743-752

Kawasaki H, Shimada H, Nakura H, Sugimura H, Tomonaga M 1987 Peculiar pattern of glial fibrillary acidic protein production in extracranial metastatic tumor cells of malignant astrocytoma. Acta Neuropathologica 74:89-91

Kendal WS, Wang R-Y, Hsu TC, Frost P 1987 Rate of generation of major karyotypic abnormalities in relationship to the metastatic potential of B16 murine melanoma. Cancer Research 47:3835-3841

Kennedy PGE, Watkins BA, Thomas DGT, Noble MD 1987 Antigenic expression by cells derived from human glioma does not correlate with morphological classification. Neuropathology and Applied Neurobiology 13:327-347

Kernohan JW, Mabon RF, Svien HJ, Adson AW 1949 A simplified classification of the gliomas. Proceedings of Staff Meetings Mayo Clinic 24:71-75

Khan P, Shin SI 1979 Cellular tumorigenicity in nude mice: test of associations among loss of cell-surface fibronectin, anchorage independence, and tumor-forming ability. Journal of Cell Biology 82:1-16

Kim SU, Moretto G, Shin DH 1985 Expression of Ia antigens on the surface of human oligodendrocytes and astrocytes in culture. Journal of Neuroimmunology 10:141-149

Kimmel DW, Shapiro JR, Shapiro WR 1987 In-vitro drug sensitivity testing in human gliomas. Journal of Neurosurgery 66:161-171

Kinzler KW, Bigner SH, Bigner DD, Trent JM, Law ML, O'Brien SJ, Wong AJ, Vogelstein B 1987 Identification of an amplified, highly expressed gene in a human glioma. Science 236:70-73

Kishikawa M, Tsuda N, Fujii H, Nishimori I, Yokoyama H, Kihara M 1986 Glioblastoma with sarcomatous component associated with myxoid change. Acta Neuropathologica 70:44-52

Klagsbrun M, Knighton D, Folkman J 1976 Tumor angiogenesis activity in cells grown in tissue culture. Cancer Research 36:110-113

Kleinsmith LJ, Pierce GB 1964 Multipotentiality of single embryonal carcinoma cells. Cancer Research 24:1544-1551

Ko HS, Fu SM, Winchester RJ, Yu DTY, Kunkel HG 1979 Ia determinants on stimulated human T lymphocytes. Journal of Experimental Medicine 150:246-255

Koch AE, Polverini PJ, Leibovich SJ 1986 Induction of neovascularisation by activated human monocytes. Journal of Leukocyte Biology 39:233-238

Kochi N, Tani E, Morimura T, Itagaki T 1983 Immunohistochemical study of fibronectin in human glioma and meningioma. Acta Neuropathologica 59:119-126

Kochi N, Budka H 1987 Histiocytic cells contribute to gliosarcoma. Acta Neuropathologica 73:124-130

Kohga S, Harvey SR, Weaver RM, Markus G 1985 Localisation of plasminogen activators in human colon cancer by immunoperoxidase staining. Cancer Research 45:1787-1796

Kopelovich L, Pfeffer LM, Bias N 1979 Growth characteristics of human skin fibroblasts in-vitro: a simple experimental approach for the identification of hereditary adenomatosis of the colon and rectum. Cancer 43:218-223

Krajewski AS, Guy K, Dewar AE, Cossar D 1985 Immunohistochemical analysis of human MHC class II antigens in B-cell non-hodgkin's lymphomas. Journal of Pathology 145:185-194

Kreipe H, Radzun H-J, Parwaresch MR 1986 Phenotypic differentiation patterns of human monocyte/macrophage system. Histochemical Journal 18:441-450

Kufe DW, Nadler L, Sargent L, Shapiro M, Hand P, Austin F, Colcher D, Schlom J 1983 Biological behaviour of human breast carcinoma-associated antigens expressed during cellular proliferation. Cancer Research 43:851-857

Kung PC, Lee JC, Bakay L 1969 Vascular invasion by glioma cells in man: an electron microscopic study. Journal of Neurosurgery 31:339-345

Kyner D, Christman J, Acs G, Silagi S, Newcomb EW, Silverstein SC 1978 Cocultivation of tumorigenic mouse melanoma cells with cells of non-tumorigenic subclone inhibits plasminogen activator expression by the melanoma cells. Journal of Cellular Physiology 95:159-167

Lachman LB, Brown DC, Dinarello CA 1987 Growth-promoting effect of recombinant interleukin 1 and tumor necrosis factor for a human astrocytoma cell line. Journal of Immunology 138:2913-2916

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Laerum OD, Haugen A, Bock E, Rosengren L, Haglid K 1985 Differentiation markers (S-100, GFAP, NSE, and D2) in fetal rat brain cells during malignant transformation in cell culture. Journal of Neuro-Oncology 3:137-146

Lampert IA, Kirkland S, Farrel S, Borysiewicz LK 1985 HLA-DR expression in a human colonic carcinoma cell line. Journal of Pathology 146:337-344

Lampson LA, Hickey WF 1986 Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from histologically normal to that showing different levels of glial tumour involvement. Journal of Immunology 136:4054-4062

Lancet 1986 Growth factors and malignancy. Lancet ii:317-318

Lancet 1987 Gene amplification in malignancy. Lancet i:839-840

Lashford LS, Davies AG, Richardson RB, Bourne SP, Bullimore JA, Eckert H, Kemshead JT, Coakham HB 1988 A pilot study of 131I monoclonal antibodies in the therapy of leptomeningeal tumors. Cancer 61:857-868

Laug WE, Tokes ZA, Benedict WF, Sorgente N 1980 Anchorage independent growth and plasminogen activator activator production by bovine endothelial cells. Journal of Cell Biology 84:281-293

Laug WE, Weinblatt ME, Jones PA 1985 Endothelial cells degrade extracellular matrix matrix proteins produced in-vitro. Thrombosis and Haemostasis 54:498-502

Lauro GM, Lorenzo ND, Grossi M, Maleci A, Guidetti B 1986 Prostaglandin E2 as an immunomodulating factor released in-vitro by human glioma cells. Acta Neuropathologica 69:278-282

Layton MG, Franks LM 1984 Heterogeneity in a spontaneous mouse lung carcinoma: selection and characterisation of stable metastatic variants. British Journal of Cancer 49:415-421

Lazarides E 1982 Intermediate filaments: a chemically heterogeneous developmentally regulated class of protein. Annual Review of Biochemistry 51:219-250

Leader M, Collins M, Patel J, Henry K 1986 Staining for factor VIII related antigen and Ulex europaeus agglutinin I (UEA-I) in 230 tumours. An assessment of their specificity for angiosarcoma and Kaposi's sarcoma. Histopathology 10:1153-1162

Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively DM, Nuseir N 1987 Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. Nature 329:630-632.

Levin EG, Loskutoff DJ 1982 Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activator. Journal of Cell Biology 94:631-636

Libermann TA, Razon N, Bartal AD, Yarden Y, Schlessinger J, Soreq H 1984 Expression of epidermal growth factor receptors in human brain tumors. Cancer Research 44:753-760

Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J 1985 Amplification and overexpression of the EGF receptor gene in primary human glioblastomas. Journal of Cell Science (Suppl) 3:161-172

Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J 1985a Amplification, enhanced expression and possible rearrangement of the EGF receptor gene in primary human brain tumours of glial origin. Nature 313:144-147

Libermann TA, Friesel R, Jaye M, Lyall RM, Westermark B, Drohan W, Schmidt A, Maciag T, Schlessinger J 1987 An angiogenic growth factor is expressed in human glioma cells. EMBO Journal 6:1627-1632

Liesi P, Kirkwood T, Vaheri A 1986 Fibronectin is expressed by astrocytes cultured from embryonic and early postnatal rat brain. Experimental Cell Research 163:175-185

Lim R, O'Connell MM 1982 Effect of Glia Maturation Factor on glial fibrillary acidic protein and fibronectin: a comparative study on glioblasts and fibroblasts using immunofluorescence. Developmental Brain Research 5:29-39

Lim R, Hicklin DJ, Ryken TC, Han X-M, Liu K-N, Miller JF, Baggenstoss BA 1986 Suppression of glioma growth in vitro and in vivo by glia maturation factor. Cancer Research 46:5241-5247

Lindgren A, Westermark B, Ponten J 1975 Serum stimulation of stationary human glia and glioma cells in culture. Experimental Cell Research 95:311-319

Little D, Said JW, Siegel RJ, Fealy M, Fishbein MC 1986 Endothelial markers in vascular neoplasms: an immunohistochemical study comparing factor VIII-related antigen, blood group specific antigens, 6-keto-PGF1 alpha, and Ulex europaeus 1 lectin. Journal of Pathology 149:89-95

Liwnicz BH, Wu SZ, Tew JM 1987 The relationship between the capillary structure and hemorrrhage in gliomas. Journal of Neurosurgery 66:536-541

Loeb L, Springate CF, Battula N 1974 Errors in DNA replication as a possible basis of malignant change. Cancer Research 34:2311-2321

Lolait, SJ, Harmer JH, Auteri G, Pedersen JS, Toh BH 1983 Expression of glial fibrillary acidic protein, actin, fibronectin, and factor VIII antigen in human astrocytomas. Pathology 15:373-378

Loskutoff DJ, Edgington TS, 1977 Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. Proceedings of the National Academy of Sciences USA 74:3903-3907

Lotem J, Sachs L 1983 Coupling of growth and differentiation in normal myeloid precursors and the breakdown of this coupling in leukemia. International Journal of Cancer 32:127-134

Lowenstein WR 1979 Junctional intercellular communication and the control of growth. Biochimica et Biophysica Acta 560:1-65

Lubitz W, Westermark B, Peterson PA 1980 Surface glycoproteins of normal and neoplastic glia cells in culture. International Journal of Cancer 25:53-58

Ludwig CL, Smith MT, Godfrey AD, Armbrustmacher VW 1986 A clinicopathological study of 323 patients with oligodendrogliomas. Annals of Neurology 19:15-21

Lumsden CE 1971 The study by tissue culture of tumours of the nervous system. In Pathology of tumours of the nervous system. Russell DS and Rubinstein LJ. 3rd Edition. Arnold, London

Lye RH, Elstow SF, Weiss JB 1986 Neovascularisation of intracranial tumours. in Biology of Brain Tumour, Walker MD, Thomas DGT (eds), Martinus Nijhoff, Boston. pp61-66

McAuslan BR, Hoffman H 1979 Endothelium stimulating factor from Walker carcinoma cells. Relation to tumor angiogenic factor. Experimental Cell Research 119:181-190

McAuslan BR, Hannan GN, Reilly W, Stewart FHC 1980 Variant endothelial cells. Fibronectin as a transducer of signals for migration and neovascularisation. Journal of Cellular Physiology 104:177-186

McComb RD, Jones TR, Pizzo SV, Bigner DD 1982 Immunohistochemical detection of Factor VIII/von Willebrand Factor in hyperplastic endothelial cells in glioblastoma and mixed glioma-sarcoma. Journal of Neuropathology and Experimental Neurology 41:479-489

McGurrin JF, Doria MI, Dawson PJ, Karison T, Stein HO, Franklin WA 1987 Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of the breast. Cancer 59:1744-1750

McKeever PE, Smith BH, Taren JA, Wahl RL, Kornblith PL, Chronwall BM 1987 Products of cells cultured from gliomas. VI. Immunofluorescent, morphometric, and ultrastructural characterization of two different cell types growing from explants of human gliomas. American Journal of Pathology 127:358-372

McLean JS, Frame MC, Freshney RI, Vaughan PFT, Mackie AE, Singer I 1986 Phenotypic modification of human glioma and non-small cell lung carcinoma glucocorticoids and other agents. Anticancer Research 6:1101-1106

Mahaley MS, Brooks WH, Roszman TL, Bigner DD, Dudka L, Richardson S 1977 Immunobiology of the cellular and humoral general immune competence of brain tumor patients. Journal of Neurosurgery 46:467-476

Maiorana A, Gullino PM 1978 Acquisition of angiogenic capacity and neoplastic transformation in the rat mammary gland. Cancer Research 38:4409-4413

Manoury P 1977 Establishment and characterisation of 5 human cell lines derived from a series of 50 primary intracranial tumours. Acta Neuropathologica 39:33-41

Mareel MM 1983 Invasion in vitro. Cancer Metastasis Reviews 2:201-218

Markus G, Kohga S, Camiolo SM, Madeja JM, Ambrus JL, Karaousis C 1984 Plasminogen activators in human malignant melanoma. Journal of the National Cancer Institute 72:1213-1222

Marshall CJ, Humphries KC, Pollack RE 1978 Microfilament bundles, LETS protein and growth control in somatic cell hybrids. Journal of Cell Science 33:191-204

Martin BA, Gimbrone MA, Unanue ER, Cotran RS 1981 Stimulation of non-lymphoid mesenchymal cell proliferation by a macrophage-derived growth factor. Journal of Immunology 126:1510-1515

Marushige Y, Raju NR, Marushige K, Koestner A 1987 Modulation of growth and of morphological characteristics in glioma cells by Nerve Growth Factor and Glia Maturation Factor. Cancer Research 47:4109-4115

Massa PT, Schimpl A, Wechker E, ter Meulen V 1987 Tumor necrosis factor amplifies measles virus-mediated Ia induction on astrocytes. Proceedings of the National Academy of Sciences USA 84:7242-7245

Masuzawa T, Saito K, Shimabukuro H, Iwasa H, Sato F 1985 Chronic encapsulated hematomas in the brain. Acta Neuropathologica 66:24-28

Mehta PM Bertram JS, Loewenstein WR 1986 Growth inhibition of transformed cells correlates with their junctional communication with normal cells. Cell 44:187-196

Merchant RE, Fontana A, Nelson AT, Young HF 1986 Mitogen-activated lymphocytes of normals and glioma patients produce factors with anti-glioblastoma activity in vitro. Journal of Neuroimmunology 11:1-14

Merchant RE, Ransom JH, Young HF 1986a Leukoregulin inhibits the growth of human glioblastoma in vitro. Journal of Neuroimmunology 13:31-45

Miescher S, Whiteside TL, de Tribolet N, von Fliedner V 1988 In situ characterisation, clonogenic potential, and antitumor cytolytic activity of T lymphocytes infiltrating human brain cancers. Journal of Neurosurgery 68:438-448

Miyagami M, Smith BH, McKeever PE, Chronwall BM, Greenwood MA, Kornblith PL 1987 Immunocytochemical localization of factor VIII-related antigen in tumors of the human central nervous system. Journal of Neuro-Oncology 4:269-285

Molne J, Jortso E, Smeds S, Ericson LE 1987 Vascularization of normal human thyroid tissue transplanted to nude mice. Experimental Cell Biology 55:104-114

Momburg F, Degener T, Bacchus E, Moldenhauer G, Hammerling GJ, Moller P 1986 Loss of HLA-A,B,C and de novo expression of HLA-D in colorectal cancer. International Journal of Cancer 37:179-184

Moore M, Ghosh AK, Johnston D 1986 Expression of MHC Class II products on human colorectal cancer. An immunohistological and flow cytometric study. Journal of Immunogenetics 13:201-209

Moore SA, Schelper RL, Hart MN, Robinson RA, Whitters E 1986a Glomeruloid hyperplasia in glioblastoma: origin in smooth muscle cells. Journal of Neuropathology and Experimental Neurology 45:327

Morantz RA, Feigin I, Ransohoff J 1976 Gliosarcoma; a clinical and pathological survey of 24 cases. Journal of Neurosurgery 45:398-408

Morantz RA, Wood GW, Foster M, Clark M, Gollahon K 1979 Macrophages in experimental and human brain tumors. Journal of Neurosurgery 50:305-311

Morgan D, Freshney RI, Darling JL, Thomas DGT, Celik F 1983 Assay of anticancer drugs in tissue culture: cell cultures of biopsies from human astrocytoma. British Journal of Cancer 47:205-214

Mork SJ, Halvorsen TB, Lindegaard K-F, Eide GE 1986 Oligodendroglioma. Histologic evaluation and prognosis. Journal of Neuropathology and Experimental Neurology 45:65-78

Morson B 1974 The polyp-cancer sequence in the large bowel. Proceedings of the Royal Society of Medicine 67:451-457

Morrison RS, De Vellis J, Lee YL, Bradshaw RA, Eng LF 1985 Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes. Journal of Neuroscience Research 14:167-76

Mosher DF, Furcht LT 1981 Fibronectin : review of its structure and possible functions. Journal of Investigative Dermatology 77:175-180

Motoi M, Yoshino T, Hayashi K, Nose S, Horie Y, Ogawa K 1985 Immunohistochemical studies on human brain tumors using anti-Leu 7 monoclonal antibody in paraffin-embedded specimens. Acta Neuropathologica 66:75-77

Mullins DE, Rorhlich ST 1983 The role of proteinases in cellular invasiveness. Biochimica et Biophysica Acta 695:177-214

Munzarova M, Kovarik J 1987 Is cancer a macrophage-mediated autoaggressive disease? Lancet i:952-954

Nagashima T, Hoshino T, Cho KG 1987 Proliferative potential of vascular elements in human glioblastoma multiforme. Acta Neuropathologica 73:301-305

Nathan CF 1987 Secretory products of macrophages. Journal of Clinical Investigation 79:319-326

Nelson DF, Nelson JS, Davis DR, Chang CH, Griffin TW, Pajak TJ 1985 Survival and prognosis of patients with astrocytoma with atypical or anaplastic features. Journal of Neuro-Oncology 3:99-103

Newcomb EW, Silverstein SC, Silagi S 1978 Malignant mouse melanoma cells do not form tumors when mixed with cells of a non malignant subclone: relationships between plasminogen activator expression by the tumor cells and the host's immune response. Journal of Cellular Physiology 95:169-177

Nister M, Betsholtz C, Bywater M, Pettersson M, Heldin C.-H and Westermark B 1985 Clonal variation of c-sis mRNA levels, PDGF production and binding of 125I-PDGF in human glioma cells. Anticancer Research 5:628

Nister M, Heldin C-H, Westermark B 1986 Clonal variation in the production of a platelet-derived growth factor-like protein and expression of corresponding receptors in a human malignant glioma. Cancer Research 46:332-340

Normann SJ 1985 Macrophage infiltration and tumour progression. Cancer Metastasis Reviews 4:277-291

Nowell PC 1986 Mechanisms of tumor progression. Cancer Research 46:2203-2207

Oberc-Greenwood MA, Muul LM, Gately MK, Kornblith PL, Smith BH 1986 Ultrastructural features of the lymphocyte-stimulated halos produced by human glioma-derived cells in vitro. Journal of Neuro-Oncology 3:387-396

Ogawa K, Hamaya K, Fujii Y, Matsuura K, Endo T 1969 Tumor induction adenovirus type 12 and its target cells in the central nervous system. Gann 60:383-392

Ogston D 1983 The physiology of haemostasis. Croom Helm, London

Oktar N, Darling JL, Thomas DGT 1987 An experimental trial of cyclic nucleotides on multicellular spheroids derived from human brain tumours. Journal of Neuro-Oncology 5:83-89

Onda K, Tanaka R, Washiyama K, Takeda N, Kumanishi T 1988 Correlation of DNA ploidy and morphological features of human glioma cell cultures with the establishment of cell lines. Acta Neuropathologica 76:433-440

Ooi B S, MacCarthy EP, Hsu A, Ooi YM 1983 Human mononuclear modulation of endothelial cell proliferation. Journal of Laboratory and Clinical Medicine 102:428-433

Ozanne B, Shum A, Richards CS, Casells D, Grossman D, Trent J, Gusterson B, Hendler F 1985 Evidence for an increase of EGF receptors in epidermoid malignancies. in Cancer Cells 3. Growth factors and transformation. Feramisco J, Ozanne B, Stiles C (eds) Cold Spring Harbor Laboratory, USA. pp41-49

Ozanne B, Richards CS, Hendler F, Burns D, Gusterson B 1986 Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. Journal of Pathology 149:9-14

Padgett BL, Walker DL, Zurhein GM, Varakis JN 1977 JC papovavirus neurooncogenicity of strains of JC virus on human polyoma virus in newborn syrian hamsters. Cancer Research 37:718-720

Paetau A 1988 Glial fibrillary acidic protein, vimentin and fibronectin in primary cultures of human glioma and fetal brain. Acta Neuropathologica 75:448-456

Paetau A, Mellstrom K, Vaheri A, Haltia M 1980 Distribution of a major connective tissue protein, fibronectin, in normal and neoplastic human nervous tissue. Acta Neuropathologica 51:47-51

Paetau A, Mellstrom K, Westermaark B, Dahl D, Haltia M, Vaheri A 1980a Mutually exclusive expression of fibronectin and glial fibrillary acidic protein in cultured brain cells Experimental Cell Research 129:337-344

Paine JT, Handa H, Yamasaki T, Yamashita J, Miyatake S 1986 Immunohistochemical analysis of infiltrating lymphocytes in central nervous system tumors. Neurosurgery 18:766-772

Palma L, DiLorenzo N, Guidetti B 1978 Lymphocytic infiltrates in primary glioblastomas and recidivous gliomas. Journal of Neurosurgery 49:854-861

Palma L, Mochese V, Galli E, Barbieri C, Lombardi VRM, Rossi P 1987 Immunological studies in patients with central nervous system tumors. Journal of Neuro-Oncology 5:29-35

Parmiani GE, Fossati G, Taramelli D, Anichini A, Balsari A, Gambacorti-Passerini C, Sciorelli G, Cascinelli N 1985 Autologous cellular immune response to primary and metastatic human melanoma and its regulation by DR antigens expressed on tumour cells. Cancer Metastasis Reviews 4:7-26

Patterson BJ, Robinson JB, Pinkerton PH 1985 Hematopoietic cell surface markers on metastatic small cell carcinoma detected with monoclonal antibodies. American Journal of Clinical Pathology 84:159-165

Pauli BU, Kellen JA, Ng R 1987 Correlation of fibrinolytic activity with invasion and metastasis of R3230 AC rat mammary carcinoma cell lines. Invasion and Metastasis 7:158-171

Pearlstein E, Hynes RO, Franks LM, Hemming VJ 1976 Surface proteins and fibrinolytic activity of cultured mammalian cells. Cancer Research 36:1475-1480

Pena CE, Felter R 1973 Ultrastructure of a composite glioma-sarcoma of the brain. Acta Neuropathologica 23:90-94

Perez M, Cabrera T, Lopez-Nevot MA, Gomez M, Peran F, Ruiz-Cabello F, Garrido F 1986 Heterogeneity of the expression of Class I and II HLA antigens in human breast carcinoma. Journal of Immunogenetics 13:247-253

Pertuiset B, Dougherty D, Cromeyer C, Hoshino T, Berger M, Rosenblum ML 1985 Stem cell studies of human malignant brain tumors. Part 2: Proliferation kinetics of brain-tumor cells in vitro in early passage cultures. Journal of Neurosurgery 63:426-432

Phillips JP, Eremin O, Anderson JR 1982 Lymphoreticular cells in human brain tumours and in normal brain. British Journal of Cancer 45:61-69

Piguet V, Carrel S, de Tribolet N 1986 Modulation of HLA-DR expression and growth inhibition on glioma cells: heterogeneous responses to interferon-gamma. in Biology of Brain Tumour, Walker MD, Thomas DGT (eds), Martinus Nijhoff, Boston. pp509-515

Piguet V, Carrel S, Diserens A-C, Mach J-P, De Tribolet N 1986a Heterogeneity of the induction of HLA-DR expression by human immune interferon on glioma cell lines and their clones. Journal of the National Cancer Institute 76:223-228

Pilkington GJ, Knott JCA, Clarke TM, Edwards A 1987 Morphological, antigenic and flow cytometric evaluation of corticosteroid-treated glioma cells in vitro. Anticancer Research 7:877

Plagemann PGW, Estensen RD, 1980 Lack of correlation between effects of tumor promoter TPA on plasminogen activator production, phosphatidyl choline synthesis, and hexose transport in mammalian cell culture systems. Journal of Cellular Physiology 104:105-119

Polverini PJ, Cotran R, Gimbrone MA, Unanue ER 1977 Activated macrophages induce vascular proliferation. Nature 269:804-806

Polverini PJ, Leibovich SJ 1984 Induction of neovascularisation in vivo and endothelial proliferation in vitro by tumor-associated macrophages. Laboratory Investigation 51:635-642

Ponten J, Westermark B, Hugosson R 1969 Regulation of proliferation and movement of human glia-like cells in culture. Experimental Cell Research 58:393-400

Ponten J, Westermark B, Hugosson R 1973 Determinants for the establishment of permanent tissue culture lines from human gliomas Acta Pathologica Scandinavica sect A 81:791-805

Ponten J, Westermark B 1978 Properties of human malignant glioma cells in vitro. Medical Biology 56:184-193

Presta M, Moscatelli D, Joseph-Silverstein J, Rifkin DB 1986 Purification from a human hepatoma cell line of a basic Fibroblast Growth Factor-like molecule that stimulates capillary endothelial cell plasminogen activator activator production, DNA synthesis, and migration. Molecular and Cellular Biology 6:4060-4066

Price J, Hynes RO 1985 Astrocytes in culture synthesize and secrete a variant form of fibronectin. Journal of Neuroscience 8:2205-2211

Pulver M, Carrel S, Mach JP, De Tribolet N 1987 Cultured human fetal astrocytes can be induced by interferon-gamma to express HLA-DR. Journal of Neuroimmunology 14:123-33

Quigley JP 1976 Association of a protease plasminogen activator with a specific membrane fraction isolated from transformed cells. Journal of Cell Biology 71:472-486

Quindlen EA, Bucher AP 1987 Correlation of tumor plasminogen activator with peritumoral edema. Journal of Neurosurgery 66:729-733

Raemaekers FCS, Puts JJG, Moesker O, Kant A, Huysmans A, Haag D, Jap PHK, Herman C, Voojis GP 1983 Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumors; an overview. Histochemical Journal 15:691-713

Raff MC, Miller RH, Noble M 1983 A glial progenitor cell that develops in-vitro into an oligodendrocyte or an astrocyte depending on culture medium. Nature 303:390-396

Rajaraman R, Westermark B, Vaheri A, Ponten J 1978 Immunofluorescence studies on fibronectin distribution patterns during adhesion, deformation and spreading of human glial and glioma cells Annals of the New York Academy of Sciences 312:444-449

Real FX, Fliegel B, Houghton AN 1988 Surface antigens of human melanoma cells cultured in serum-free medium: induction of expression of major histocompatibility complex Class II antigens. Cancer Research 48:686-693.

Reddy AL, Fialkow PJ 1980 Multicellular origin of fibrosarcomas in mice induced by the chemical carcinogen 3-methylcholanthrene. Journal of Experimental Medicine 150:878-887

Reddy KNN, Kline DL 1980a Plasminogen activators. Ch.2 in Fibrinolysis, DL Kline, KNN Reddy (eds), CRC Press Florida

Reifenberger G, Szymas J, Weschler W 1987 Differential expression of glial- and neuronal-associated antigens in human tumours of the central and peripheral nervous system. Acta Neuropathologica 74:105-123

Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG 1981 Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proceedings of the National Academy of Sciences USA 78:7147-7151

Rettig WR, Chesa PG, Beresford HR, Feichert H-J, Jennings MT, Cohen J, Oettgen, Old L 1986 Differential expression of cell surface antigens and glial fibrillary acidic protein in human astrocytoma subsets. Cancer Research 46:6406-12

Rey JA, Bello MJ, De Campos JM, Kusak ME, Ramos C, Benitez J 1987 Chromosomal pattern in human malignant astrocytomas. Cancer Genetics and Cytogenetics 29:201-221

Rey JA, Bello MJ, De Campos JM, Kusak ME, Moreno S 1987a Chromosomal composition of a series of 22 low-grade gliomas. Cancer Genetics and Cytogenetics 29:223-237

Ridley A, Cavanagh JB 1971 Lymphocytic infiltration in gliomas: evidence of possible host resistance. Brain 94:117-124

Rijken DC, Wijngaards G, Zaal-de Jong M, Welbergen J 1979 Purification and partial characterisation of plasminogen activator from human uterine tissue. Biochimica et Biophysica Acta 580:140-153

Rijken DC, Wijngaards G, Welbergen J 1980 Relationship between tissue plasminogen activator and the activators in blood and vascular wall. Thrombosis Research 18:815-830

Rijken DC, Collen D 1981 Purification and characterisation of the plasminogen activator secreted by human melanoma cells in culture. Journal of Biological Chemistry 256:7035-7041

Riley PA 1981 Control of proliferation of normal and neoplastic cells in culture. in Regulation of growth in neoplasia Sherbet GV (ed) Karger Basel. pp131-198,

Ring NP, Addis BJ 1986 Thymoma: an integrated clinicopathological and immunohistochemical study. Journal of Pathology 149:327-337

Ringherz N 1950 "Grading" of gliomas. Acta Pathologica et Microbiologica Scandinavica 27:51-64

Robinson RA, Branum EL, Volkenant ME, Moses HL 1982 Cell cycle variation in 125I-labelled epidermal growth factor binding in chemically transformed cells. Cancer Research 42:2633-2638

Roessmann U, Velasco MF, Gambetti P, Autilio-Gambetti L 1983 Vimentin intermediate filaments are increased in human neoplastic astrocytes. Journal of Neuropathology and Experimental Neurology 42:309

Roitt IM, Brostoff J, Male DK 1985 The Major Histocompatibility Complex. in Immunology, Churchill Livingstone. pp4.1-4.12

Rorke LB 1987 Phenotypic classification of tumors. Journal of Neuro-Oncology 5:95-97

Rosenblum ML, Gerosa M, Dougherty DV, Reese C, Barger GR, Davis RL, Levin VA, Wilson CB 1982 Age-related chemosensitivity of stem cells from human malignant brain tumours. Lancet i:885-887

Ross R 1971 The smooth muscle cell. II Growth of smooth muscle in culture and formation of elastic fibers. Journal of Cell Biology 50:172-186.

Rossi ML, Hughes JT, Esiri MM, Coakham HB, Brownell DB 1987 Immunohistological study of mononuclear cell infiltrate in malignant gliomas. Acta Neuropathologica 74:269-277

Rossi ML, Cruz Sanchez F, Hughes JT, Esiri MM, Coakham HB 1988 Immunocytochemical study of the cellular immune response in meningiomas. Journal of Clinical Pathology 41:314-319

Rossi ML, Cruz-Sanchez F, Hughes JT, Esiri MM, Coakham HB, Moss TH 1988a Mononuclear cell infiltrate and HLA-DR expression in low grade astrocytomas. An immunohistological study of 23 cases. Acta Neuropathologica 76:281-286

Roszman TL, Brooks WH, Elliot LH 1987 Inhibition of lymphocyte responsiveness by a glial tumor cell-derived suppressive factor. Journal of Neurosurgery 67:874-879

Rovera G, O'Brien TG, Diamond L 1977 Tumor promoters inhibit spontaneous differentiation of Friend erythroleukemia cells in culture. Proceedings of the National Academy of Sciences USA 74:2894-2898

Rubin K, Tingstrom A, Hannson GK, Larsson E, Ronnstrand L, Klareskog L, Claessson-Welsh L, Heldin C-H, Fellstrom B, Terracio L 1988 Induction of B-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. Lancet i:1354-1356

Rubinstein LJ 1956 The development of contiguous sarcomatous and gliomatous tissue in intracranial tumours. Journal of Pathology and Bacteriology 71:441-459

Rubinstein LJ 1972 Tumors of the Central Nervous System. Atlas of Tumor Pathology, Second series, Fascicle 6, Armed Forces Institute of Pathology, Washington

Ruiter DJ, Bergman W, Welvaart K, Scheffer E, van Vloten WA, Russo C, Ferrone S 1984 Immunohistochemical analysis of malignant melanomas and nevocellular nevi with monoclonal antibodies to distinct monomorphic determinants of HLA antigens. Cancer Research 44:3930-3935

Ruff MR, Pert CB 1984 Small cell carcinoma of the lung: macrophage-specific antigens suggest haemopoietic stem cell origin. Science 225:1034-1036

Ruoslahti E 1984 Fibronectin in cell-adhesion and invasion. Cancer Metastasis Reviews 3:43-51

Ruoslahti E, Vaheri A, Kuusela P, Linder E 1973 Fibroblast surface antigen: a new surface protein. Biochimica et Biophysica Acta 322:352-358

Ruoslahti E, Engvall E, Hayman EG 1981 Fibronectin: current concepts of its structure and function. Collagen Research 1:95-128

Russell DS, Rubinstein LJ 1977 Pathology of tumours of the central nervous system. Fourth edition. Arnold London

Rutka JT, Giblin JR, Hoifodt HK, Dougherty DV, Bell CW, McCulloch JR, Davis RL, Wilson CB, Rosenblum ML 1986 Establishment and characterisation of a cell line from a human gliosarcoma. Cancer Research 46:5893-5902

Rutka JT, Kleppe-Hoifodt H, Emma DA, Giblin JR, Doughterty DV, McCulloch JR, DeArmond SJ, Rosenblum ML 1986a Characterization of normal human brain cultures. Evidence for outgrowth of leptomeningeal cells. Laboratory Investigation 55:71-85

Rutka JT, Giblin JR, Apodaca G, DeArmond SJ, Stern R, Rosenblum ML 1987 Inhibition of growth and induction of differentiation in a malignant human glioma cell line by normal leptomeningeal extracellular matrix proteins. Cancer Research 47:3515-3522

Rutka JT, Giblin JR, Dougherty DY, Liu HC, McCulloch JR, Bell CW, Stern RS, Wilson CB, Rosenblum ML 1987a Establishment and characterization of five cell lines derived from human malignant gliomas. Acta Neuropathologica 75:92-103

Sachs L 1984 Normal regulators, oncogenes and the reversibility of malignancy. Cancer Surveys 3:219-228

Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL 1987 Epidermal-Growth-Factor receptor status as predictor of early recurrence of and death from breast cancer. Lancet i:1398-1402

Saison M, Van Leuven F, Cassiman JJ, Van der Berghe H 1983 Evidence for a role of fibronectin and other cell surface components in cell-cell adhesion of human fibroblasts. Experimental Cell Research 143:237-245

Sasaki K, Murakami T, Kawasaki M, Takahishi M 1987 The cell cycle associated change of Ki67 reactive nuclear antigen expression. Journal of Cellular Physiology 133:579-584.

Schachner M, Schoonmaker G, Hynes RO 1978 Cellular and subcellular localization of LETS protein in the nervous system. Brain Research 158:149-158

Schiffer D, Giordana MT, Mauro A, Migheli A 1984 GFAP, FVIII/RAg, laminin and fibronectin in gliosarcomas: an immunohistochemical study. Acta Neuropathologica 63:108-116

Schiffer D, Giordana MT, Mauro A, Migheli A, Germano I, Giaccone G 1986 Immunohistochemical demonstration of vimentin in human cerebral tumours. Acta Neuropathologica 70:209-219

Schiffer D, Giordana MT, Migheli A, Giaccone G, Pezzotta S, Mauro A 1986a Glial fibrillary acidic protein in the experimental glial reaction of the rat brain. Brain Research 374:110-118

Schiffer D, Chio A, Giordana MT, Leone M, Soffietti R 1988 Prognostic value of histologic factors in adult cerebral astrocytoma. Cancer 61:1386-1393.

Schindler A-M, Amaudruz MA, Kocher O, Riotton G, Gabbiani G 1982 Desmosomes and gap-junctions in various epidermoid preneoplastic and neoplastic lesions of the cervix uteri. Acta Cytologica 26:466-470

Schnyder B, Weber E, Fierz W, Fontana A 1986 On the role of astrocytes in polyclonal T cell activation. Journal of Neuroimmunology 10:209-218

Schoefl GI 1963 Studies on inflammation III. Growing capillaries: their structure and permeability. Virchows Archives of Pathology Anatomy and Physiology 337:97-141

Schor AM, Schor SL, Weiss JB, Brown RA, Kumar S, Phillips P 1980 Stimulation by a low-molecular weight angiogenic factor of capillary endothelial cells in culture. British Journal of Cancer 41: 790-799

Schor SL, Schor AM, Bazill GW 1981 The effects of fibronectin on the migration of human foreskin fibroblast and syrian hamster melanoma cells into three dimensional gels of native collagen fibres. Journal of Cell Science 48:301-314

Schor AM, Schor SL 1983 Tumour angiogenesis. Journal of Pathology 141:385-413

Schreiber AB, Winkler ME, Derynck R 1986 Transforming growth factor alpha: a more potent angiogenic mediator than Epidermal Growth Factor. Science 232:1250-1253

Schwarting R, Stein H, Wang CY 1985 The monoclonal antibodies antiS-HCL 1 (antiLeu-14) and antS-HCL 3 (antiLeu-M5) allow the diagnosis of hairy cell leukaemia. Blood 65:974-983

Schwarz I, Feigin I 1963 Sarcoma arising in metastatic carcinoma of the brain. Acta Neuropathologica 3:74-78

Seligman AM, Shear MJ 1939 Studies in carcinogenesis. VIII Experimental production of brain tumors in mice with methyl cholanthrene. American Journal of Cancer 37:364-395

Siegel, S 1956 Non-parametric statistics for the behavioural sciences. McGraw-Hill, Kagakusha, Japan

Shapiro JR 1986 Biology of gliomas: heterogeneity, oncogenes, growth factors. Seminars in Oncology 13:4-15

Shapiro JR, Yung W-KA, Shapiro WR 1981 Isolation, karyotype and clonal growth of heterogeneous subpopulations of human malignant gliomas. Cancer Research 41:2349-2359

Shapiro JR, Shapiro WR 1985 The subpopulations and isolated cell types of freshly resected high grade human gliomas: their influence on the tumor's evolution in-vivo and behaviour and therapy in-vitro. Cancer Metastasis Reviews 4:107-124

Sharp G, Osborn M, Weber K 1982 Occurrence of two different intermediate filament proteins in the same filament in situ within a human glioma cell line. Experimental Cell Research 141:385-395

Sherbet GV, Tindle ME, Stidolph S 1982 Fibronectin in human astrocytomas grown in tissue culture. Anticancer Research 2:251-254

Shingleton HM, Richart RM, Wiener J, Spiro D 1968 Human cervical intraepithelial neoplasia - fine structure of dysplasia and carcinoma-in-situ. Cancer Research 28:695-706

Shingleton HM, Wilbank GD 1974. Fine structure of human cervical intraepithelial neoplasia in vivo and in vitro. Cancer 33:981-989

Shiurba RA, Vogel H, Eng LF, Urich H, Horoupian D 1988 The epidermal growth factor receptor in meningiomas: an immunohistochemical study. Journal of Neuropathology and Experimental Neurology 47:376

Sholley MM, Ferguson GP, Seibel HR, Montour JL, Wilson JD 1984 Mechanisms of neovascularisation. Vascular sprouting can occur without proliferation of endothelial cells. Laboratory Investigation 51:624-634

Sidky YA, Borden EC 1987 Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. Cancer Research 47:5155-5161.

Simnett JD 1981 Regulation of growth and cell division in the whole organism. in Regulation of growth in neoplasia Sherbet GV (ed), Karger Basel. pp1-51,

Slowik F, Jellinger K, Gaszo L, Fischer J 1985 Gliosarcomas: histological, immunohistochemical, ultrastructural, and tissue culture studies. Acta Neuropathologica 67:201-210

Smith MT, Ludwig CI, Godfrey AD, Armbrustmacher VW 1983 Grading of oligodendrogliomas. Cancer 52:2107-2114

Smith MEF, Bodmer WF, Bodmer JG 1988 Selective loss of HLA-A,B,C locus products in colorectal adenocarcinoma. Lancet i:823-824

Soberano ME, Ong EB, Johnson AJ, Levy M, Schoellmann G 1976 Purification and characterization of two forms of urokinase. Biochimica et Biophysica Acta 445:763-773

Soreq H, Miskin R 1981 Plasminogen activator in the rodent brain. Brain Research 216:361-374.

Spaar FW, Ahyai A, Spaar U, Gazso L, Zimmermann A 1986 Flow-cytophotometry of nuclear DNA in biopsies of 45 human gliomas and after primary culture in vitro. Clinical Neuropathology 5:157-175

Stavrou D, Anzil AP, Weidenbach W, Rodt H 1977 Immunofluorescence study of lymphocytic infiltration in gliomas. Identification of T lymphocytes. Journal of the Neurological Sciences 33:275-282

Stavrou D, Mellert W, Mellert U, Keiditsch E, Bise K, Mehraein P 1986 Growth inhibition of experimental glioma grafts by monoclonal antibody treatment. Journal of Cancer Research and Clinical Oncology 112:111-118

Steck PA, Gallick GE, Maxwell SA, Kloetzer WS, Arlinghaus RB, Moser RP, Gutterman JU, Yung W-KA 1986 Expression of epidermal growth factor receptor and associated glycoprotein on cultured human brain tumour cells. Journal of Cellular Biochemistry 32:1-10

Stein AA, Mauro J, Thibodeau L, Alley R 1969 The histogenesis of cardiac myxomas: relation to other proliferative diseases of subendothelial vasoform reserve cells. In Pathology Annual, Vol 4, SC Sommers (ed). Appleton-Century-Crofts, New York. pp293-312,

Steinberg MS 1970 Does differential adhesion govern self assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. Journal of Experimental Zoology 173:395-434

Stevens A, Kloter I, Roggendorf W 1988 Inflammatory infiltrates and natural killer cell presence in human brain tumours. Cancer 61:738-743

Stephenson TJ, Griffiths DWR, Mills PM 1986 Comparison of Ulex europaeus I lectin binding and factor VIII-related antigen as markers of vascular endothelium in follicular carcinoma of the thyroid. Histopathology 10:251-260

Stoscheck CM, King LE 1986 Role of epidermal growth factor in carcinogenesis. Cancer Research 46:1030-1037

Studer A, De Tribolet N, Diserens AC, Gaide AC, Matthieu JM, Carrel S, Stavrou D 1985 Characterisation of four human malignant glioma cells lines. Acta Neuropathologica 66:208-217

Takahashi H, Herlyn D, Atkinson B, Powe J, Rodeck U, Alavi A, Bruce DA, Koprowski H 1987 Radioimmunodetection of human glioma xenografts by monoclonal antibody to epidermal growth factor receptor. Cancer Research 47:3847-3850

Takahashi T, Yamaguchi T, Kitamura K, Suzuyama H, Honda M, Yokota T, Kotanagi H, Takahashi M, Hashimoto Y 1988 Clinical application of monoclonal antibody-drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. Cancer 61:881-888

Takashima s, Kogha M, Tanaka K 1969 Fibrinolytic activity of human brain and cerebrospinal fluid. British Journal of Experimental Pathology 50:533-539

Takeuchi J, Barnard RO 1976 Perivascular lymphocytic cuffing in astrocytomas. Acta Neuropathologica 35:265-271

Takiguchi M, Ting JP-Y, Buessow SC, Boyer C, Gillespie Y, Frelinger JA 1985 Response of glioma cells to gamma-interferon: increase in class II RNA, protein and mixed lymphocyte reaction-stimulating ability. European Journal of Immunology 15:809-814

Tannock IF 1970 Population kinetics of carcinoma cells, capillary endothelial cells and fibroblasts in a transplanted mouse mammary tumour. Cancer Research 30:2470-2476

Taylor S, Folkman J 1982 Protamine is an inhibitor of angiogenesis. Nature 297:307-312

Thomas TL, Stolley PD, Stemhagen A, Fontham ETH, Bleeker ML, Stewart PA, Hoover RN 1987 Brain tumor mortality risk among men with electrical and electronic jobs: a case control study. Journal of the National Cancer Institute 79:233-238

Thompson DM, Gill GN 1985 The EGF receptor: structure, regulation and potential role in malignancy. Cancer Surveys 4:767-788

Thompson WD, Shiach KJ, Fraser RA, McIntosh LC, Simpson JG 1987 Tumours acquire their vasculature by vessel incorporation,not vessel ingrowth. Journal of Pathology 151:323-332

Tiku ML Tomasi ML 1985 Enhancement of endothelial plasminogen activator synthesis by lymphokines. Transplantation 40:293-298

Todaro GJ, DeLarco J 1978 Growth factors produced by sarcoma virus-transformed cells. Cancer Research 38:4147-4154

Todd AS 1959 The histological localisation of fibrinolysin activator. Journal of Pathology and Bacteriology 78:281-283

Toole BP 1981 Glycosaminoglycans in morphogenesis. Ch.9 in Cell Biology of the Extracellular Matrix, ED Hay (ed). Plenum Press, London. pp259-294

Toshniwal PK, Firestone SL, Barlow GH, Tiku ML 1987 Characterisation of astrocyte plasminogen activator. Journal of the Neurological Sciences 80:277-287

Toshniwal PK, Tiku ML, Tiku K Skosey JL 1987a Secretion of plasminogen activator by cerebral astrocytes and its modulation. Journal of the Neurological Sciences 80:307-321

Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences USA 76:4350-4354

Townes P, Holtfreter J 1955 Directed movements and selected adhesion of embryonic amphibian cells. Journal of Experimental Zoology 128:53-120

Traugott U, Lebon P 1988 Interferon-gamma and Ia antigens are present on astrocytes in active chronic multiple sclerosis. Journal of the Neurological Sciences 257:264

Trejdosiewicz LK, Southgate J, Hodges GM, Goodman SL 1985 Micro-heterogeneous expression of peanut agglutinin-binding sites in the extracellular matrix of cultured cells. Experimental Cell Research 156:153-163

Tsukamoto Y, Helsel WE, Wahl SM 1981 Macrophage production of fibronectin, a chemoattractant for fibroblasts. Journal of Immunology 127:673-678

Tucker WS, Kirsh WM, Martinez-Hernadez A, Fink LM 1978 In-vitro plasminogen activator activity in human brain tumours. Cancer Research 38:297-302

Uegaki M, Kobayashi S, Kuramoto S, Yokoyama MM 1988 Lymphocyte subsets in patients with brain tumors. Journal of Neuro-Oncology 6:25-28

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH 1984 Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309:418-425

Unkeless JC, Gordon S, Reitch E 1974 Secretion of plasminogen activator by stimulated macrophages. Journal of Experimental Medicine 139:834-850

Vaheri A, Ruoslahti E 1974 Disappearance of a major cell-type specific glycoprotein antigen (SF) after transformation of fibroblasts by rous sarcoma virus. International Journal of Cancer 13:579-586

Vaheri A, Ruoslahti E, Westermark B, Ponten J 1976 A common cell-type specific surface antigen in cultured human glial cells and fibroblasts: loss in malignant cells. Journal of Experimental Medicine 143:64-72

Van Duinen SG, Ruiter DJ, Ferrone S 1986 Associated expression of HLA Class I and II antigens on melanoma cells in surgically removed metastases. Journal of Pathology 149:339-348

Vasiliev JM, Gelfand IM 1981 Neoplastic and normal cells in culture. Ch.14, Cambridge University Press, Cambridge. pp 265-274

Vilcek J, Palombella VJ, Henriksen-DeStefano D, Swenson C, Feinman M, Hirai M, Tsujmoto M 1986 Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. Journal of Experimental Medicine 163:632-643

Von Hanwehr RI, Hofman FM, Taylor CR, Apuzzo MLJ 1984 Mononuclear lymphoid populations infiltrating the microenvironment of primary CNS tumours. Journal of Neurosurgery 60:1138-1147

Wagoner JK, Infante PF, Apfeldorf RB 1981 Toxicity of vinyl chloride and polyvinyl chloride as seen through epidemiologic observations. In Occupational cancer and carcinogenesis, H Vainio, M Sorsa, Hemminki K eds. McGraw Hill, Washington. pp181-188

Wang N, Yu SH, Liener IE, Hebbel RP, Eaton JW, McKhann CF 1982 Characterisation of high- and low-metastatic clones derived from a methylcholanthrene-induced murine fibrosarcoma. Cancer Research 42:1046-1051

Wang E, Cairncross JG, Liem RKH 1984 Identification of glial filament protein and vimentin in the same intermediate filament system in human glioma cells. Proceedings of the National Academy of Sciences USA 81:2102-2106

Warren BA, Shubik P 1966 The growth of the blood supply to melanoma transplants in the hamster cheek pouch. Laboratory Investigation 15:464-478

Warren BA, Greenblatt M, Kommineni VRC 1972 Tumour angiogenesis: ultrastructure of endothelial cells in mitosis. British Journal of Experimental Pathology 53:216-224

Wartiovaara J, Linder E, Ruoslahti E, Vaheri A 1974 Distribution of fibroblast surface antigen: association with fibrillar structures of normal cells and loss upon viral transformation. Journal of Experimental Medicine 140:1522-1533

Waterfield MD, Mayes ELV, Stroobant P, Bennet PLP, Young S, Goodfellow PN, Banting GS, Ozanne B 1983 A monoclonal antibody to human epidermal growth factor receptor. Journal of Cellular Biochemistry 20:149-161

Weber T, Seitz RJ, Liebert VG, Gallasch E, Weschler W 1985 Affinity cytochemistry of vascular endothelium in brain tumors by biotinylated Ulex Europaeus type I lectin (UEl). Acta Neuropathologica 67:128-135

Wellington DG, Macdonald EJ, Wolf PF 1979 Cancer epidemiology. Environmental ethnic factors. Academic Press, New York, pp84-85 and 112-113

West, DC, Kumar S 1988 Endothelial cell proliferation and diabetic retinopathy. Lancet i:715-716

Westermark B 1973 The deficient density-dependent growth control of human malignant glioma cells and virus-transformed glia-like cells in culture. International Journal of Cancer 12:438-451

Westermark B 1977 Local starvation for epidermal growth factor cannot explain density-dependent inhibition of normal human glial cells. Proceedings of the National Academy of Sciences USA 74:1619-1621

Westermark B, Magnusson AS, Heldin C-H 1982 Effect of epidermal growth factor on membrane motility and cell locomotion in cultures of human clonal glioma cells. Journal of Neuroscience Research 8:491-507

Westermark B, Nister M, Heldin C-H 1985 Growth factors and oncogenes in human malignant glioma. Neurologic Clinics 3:785-799

Westphal M, Harsh GR, Rosenblum ML, Hammonds RG 1985 Epidermal growth factor receptors in the human glioblastoma cell line SF268 differ from those in epidermoid carcinoma cell line A431. Biochemistry and Biophysics Research Communications 132:284-289

Westphal M, Haensel M, Mueller D, Laas R, Kunzmann R, Rohde E, Koenig A, Hoelzel F, Hermann HD 1988 Biological and karyotypic characteristics of a new cell line derived from human gliosarcoma Cancer Research 48:731-740

White D 1981 Cell interactions and the control of development in myxobacteria populations. International Review of Cytology 72:203-228

White WF, Barlow GH, Mozen M 1966 The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry 5:2160-2169

Wigler M, Weinstein IB 1976 Tumour promoter induces plasminogen activator. Nature 259:232-233

Wikstrand CJ, Grahmann FC, McComb RD, Bigner DD 1985 Antigenic heterogeneity of human anaplastic gliomas and glioma-derived cell lines defined by monoclonal antibodies. Journal of Neuropathology and Experimental Neurology 44:229-241

Wilkinson IMS, Anderson JR, Holmes AE 1987 Oligodendroglioma: an analysis of 42 cases. Journal of Neurology Neurosurgery and Psychiatry 50:304-312

Williamson JMS, Grigor I, Smith MEF, Holgate CS, Quirke P, Bird CC 1986 Cluster differentiation antigen expression, proliferative activity and clinical stage in centroblastic centrocytic lymphomas. Journal of Pathology 150:51-59

Willis RA 1948 Pathology of Tumours. First edition , Butterworth, London. ppl40-141

Willis RA 1958 The embryonic tumours. In The borderland of embryology and pathology. Butterworth, London. pp411-415

Wilson EL, Dowdle EB 1978 Secretion of Plasminogen activator by normal, reactive and neoplastic human tissues cultured in-vitro. International Journal of Cancer 22:390-393.

Wilson EL, Becker MLB, Hoal EG, Dowdle EB 1980 Molecular species of plasminogen activators secreted by normal and neoplastic human cells Cancer Research 40:933-938

Wissler RW 1967 The arterial medial cell, smooth muscle, or multifunctional mesenchyme. Circulation 36:1-4

Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B 1987 Increased expression of the epidermal growth factor receptor gene is invariably associated with gene amplification. Proceedings of the National Academy of Sciences USA 84:6899-6903

Wood GW, Morantz RA 1979 Immunohistological evaluation of the lymphoreticular infiltrate in human CNS tumours. Journal of the National Cancer Institute 62:485-490

Wrann M, Bodmer S, De Martin R, Siepl C, Hofer-Warbinek R, Frei K, Hofer E, Fontana A 1987 T-cell suppressor factor from human glioblastoma cells is a 12.5 -kd protein closely related to transforming growth factor-beta. EMBO Journal 6:1633-1636

Wright SD, Pilkington GJ 1987 Antigenic and morphological response og glioma cells to corticosteroid treatment in vitro. Neuropathology and Applied Neurobiology 13:235

Yamada KM, Yamada SS, Pashen I 1976 Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. Proceedings of the National Academy of Sciences USA 73:1217-1221

Yamada KM, Olden K 1978 Fibronectins - adhesive glycoproteins of cell surface and blood. Nature 275:179-184

Yasuda K, Alderson T, Phillips J, Sikora K 1983 Detection of lymphocytes in malignant gliomas by monoclonal antibodies. Journal of Neurology Neurosurgery and Psychiatry 46:734-737

Yoshida J, Wakabayashi T, Kito A, Kageyama N, Murata Y, Seo H, Kojima N, Yagi K 1987 Clinical application of monoclonal antibodies against glioma-associated antigens. Progress in Experimental Tumour Research 30:40-56

Yoshimine T, Maruno M, Ushio Y, Hayakawa T, Nakajima Y, Mogami H 1988 Intermediate filaments and anaplastic change of ENU-induced gliomas: immunohistochemical study with vimentin and astroprotein (GFAP). Journal of Neuro-Oncology 5:377-385

Yung W-KA, Shapiro JR, Shapiro WR 1982 Heterogeneous chemosensitivities of subpopulations of human glioma cells in culture. Cancer Research 42:992-998

Yung W-KA, Luna M, Borit A 1985 Vimentin and glial fibrillary acidic protein in human brain tumors. Journal of Neuro-Oncology 3:35-38

Zetter BR 1980 Migration of capillary endothelial cells is stimulated by tumour-derived factors. Nature 285:41-43

Ziche M, Gullino PM 1982 Angiogenesis and neoplastic progression in vitro. Journal of the National Cancer Institute 69:483-487

Zuk JA, Walker RA 1987 Immunohistochemical analysis of HLA antigens and mononuclear infiltrates of benign and malignant breast. Journal of Pathology 152:275-285

Zulch KJ 1979 Histological typing of tumours of the central nervous system. World Health Organisation, Geneva